昭和32年7月9日 第三種郵便物認可
第46巻 第11号 昭和34年11月25日発行
(毎月1回25日発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 46, No. 11, November 25, 1959

Vol. 46, No. 11 **November, 1959**

THE JOURNAL
OF
BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

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BY
THE JAPANESE BIOCHEMICAL SOCIETY
Tokyo University, Tokyo

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PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

STUDIES ON METABOLISM OF AMIDES IN MYCOBACTERIACEAE

IV. FORMATION AND HYDROLYSIS OF HYDROXAMATE

By TOKUJI KIMURA

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(Received for publication, December 8, 1958)

Kimura and Sasakawa (*1*) discovered in their studies on fatty acid metabolism of *Mycobacterium avium*, another type of hydroxamate formation entirely different from the types known either in animal tissue or in bacteria. This reaction refers to the hydroxamate formation from free acid and hydroxylamine in the absence of ATP*, CoA, and metal ions. *n*-Valeric, *n*-butyric, and sebacic acids are the best substrates among the fatty acids tested.

This paper will report further studies on the formation and hydrolysis of hydroxamate by *M. avium* (Takeo), and also describe the purification and properties of the enzymes.

METHODS AND MATERIALS

Quantitative Analysis—The amount of the hydroxamate was electrophotometrically measured by the color of its ferric complex in accordance with the Lipmann and Tuttle method (*2*).

The protein was measured by Lowry's method (*3*) and biuret method.

Assay of Enzyme Activities—For hydroxamate formation, standard assay mixture contained in 2.5 ml., 40 μM of sodium bicarbonate, 20 μM of *n*-valerate, 1000 μM of hydroxylamine adjusted to pH 6.6, and water if necessary.

The reaction was started by the addition of the enzyme (1000 μg. or less of protein in 0.1 ml.)

The incubation was carried out for 60 minutes at 38°. The blank control, which contained all components except the substrate, produced negligible amount of hydroxamate.

For hydroxamate hydrolysis, the standard assay mixture contained in 4.0 ml., 200 μM of phosphate or Tris buffer, hydroxamate, and the enzyme. The reaction was started by the addition of the enzyme, and the incubation was carried out for 60 minutes at 38°. The amount of hydroxamate was measured before and after the incubation, the difference being taken as the quantities hydrolysed.

For the measurement of esterase activity, the rate of hydrolysis of ester was determined by the alkaline titration method (*4*) or by the hydroxamic acid method of Stern *et al.* (*5*).

Materials Used—The fatty acids were obtained from commercial sources, and distilled once. *n*-Valeric (b.p. 181–184.4°), and *n*-butyric acids were used in 0.1 *M* solution adjusted to pH 7.0. Ethyl valerate, and tributyrin were also obtained from commercial sources, and used in 0.05 or 0.01 *M* solution containing polyvinyl alcohol. Hydroxamate were prepared by the ordinary method from hydroxylamine and each of methyl valerate, methyl butyrate, methyl nicotinate, and ethyl benzoate. About 0.1 or 0.05 *M* solution was freshly prepared when used. Sodium-ATP was obtained from Biochimica A. G. (Zürich).

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* The following abbreviation will be used: ATP, adenosine triphosphate; BenzHA, benzohydroxamate; BuHA, *n*-butyrohydroxamate; CoA, coenzyme A; EDTA, ethylenediaminetetraacetate; PCMB, *p*-chloromercuribonzoate; MIA, monoiodoaceate; VHA, *n*-valerohydroxamate, NHA, nicotinohydroxamate.

Purification of the Enzyme Catalysing the Hydroxamate Formation

Preparation of Cell-free Extract—Mycobacterium avium (strain Takeo) was grown in glycerol-bouillon medium for 3 days. The washed cells were frozen and powdered by dry-ice, and then poured into 20 volumes of acetone previously cooled to −30°. After brief stirring, the cells were allowed to settle, the supernatant fluid was decanted, and filtered, and then the filter cake of the cells was desiccated in vacuo until the solvent completely evaporated.

A portion of these acetone-dried cells was then finely ground with 4 portions of quartz sand and subsequently extracted with 50 volumes of 0.02 *M* sodium bicarbonate. After standing overnight in a refrigerator, the cells were centrifuged off at 3000 r. p. m. for 30 minutes, and the supernatant fluid was used as crude extract. (Fraction A)

Preparation of Purified Enzyme—First Ammonium Sulfate Fractionation: The crude extract was brought to 20 per cent saturation with ammonium sulfate. After centrifugation the precipitate formed was discarded and the supernatant fluid was next brought to 80 per cent saturation with solid ammonium sulfate. The active material was precipitated and dissolved in 0.02 *M* sodium bicarbonate. (Fraction B)

Second Ammonium Sulfate Fractionation: Solid ammonium sulfate was added to the above enzyme solution. The fraction precipitating between 30 and 60 per cent saturation was collected. The precipitate was dissolved in 0.02 *M* sodium bicarbonate and dialysed for 4 hours. (Fraction C)

Charcoal Treatment: For the removal of nucleotides, activated charcoal (100–150 mesh) was added to the enzyme solution. After stirring for 10 minutes, the mixture was allowed to stand for 30 minutes at 0°. The charcoal was removed by centrifugation. By this procedure the enzyme contained in the supernatant fluid became negative in tests for pentose with orcinolhydrochloric acid and cysteine-sulfuric acid. (Fraction D)

Calcium Phosphate Gel Adsorption: Fraction D was adjusted to pH 6.8 and 0.2 volume of calcium phosphate gel was added to the enzyme solution. The suspension was allowed to stand for 30 minutes with occasional stirring, and then centrifuged. The supernatant fluid had no activity, and the gel was eluted with 0.1 *N* sodium hydrogenphosphate. The gel was removed by centrifugation after standing for 20 minutes.

Third Ammonium Sulfate Fractionation: The gel-eluate was fractionated as before with solid ammonium sulfate at 0°. Three fractions were collected between 30–40, 40–50, 50–60 per cent saturation respectively. The major part of the activity usually appeared in the fraction of 50–60 per cent saturation. This fraction taken up in 0.02 *M* sodium bicarbonate, was stable for several months if stored in a frozen state. (Fraction F). The purification procedure is summarized in Table I.

Purification of the Enzyme Catalysing the Hydroxamate Hydrolysis—Five grams of the acetone-dried cells were finely ground with a large amount of sea sand, and subsequently extracted with 200 ml. of 1.0 per cent sodium bicarbonate. After standing overnight in a refrigerator, the cells were centrifuged off at 10,000 r. p. m. for 20 minutes at 0°. The supernatant fluid was used as crude extract. 2.0 ml. of 1 per cent protamine sulfate solution were added to 100 ml. of the crude extract. After being stirred and standing for 20 minutes, the precipitate formed was centrifuged off at 10,000 r. p. m. for 20 minutes.

TABLE I

Summary of Purification Procedure of the Enyme Catalysing the Hydroxamate Formation

Fraction	Total volume (*ml.*)	Total protein (*mg.*)	Total units (*units*)	Specific activity (*units/mg.*)	Yield (%)
A. Crude extract	340	1340	1206	0.9	100
B. 1st ammonium sulfate fractionation	113	430	1161	2.7	96
C. 2nd ammonium sulfate fractionation	57	240	1038	4.4	86
D. Charcoal supernatant	72	140	690	5.0	57
E. Gel eluate	81	4.7	564	12.0	47
F. 3rd ammonium sulfate fractionation	5	3	52	17.2	4

Specific activity=units/mg.=μ moles of hydroxamate formed/hr./mg. protein

TABLE II

Summary of Purification of the Enzyme Hydrolysing Hydroxamate from Mycobacterium avium

Source	Fraction	Specific activity*			
		Nicotinohydroxamate (12 μM)	Benzohydroxamate (11 μM)	Valerohydroxamate (10 μM)	Butyrohydroxamate (15 μM)
M. avium	Crude extract	4.27	1.95	0.90	1.19
	Protamine supernatant	5.00	1.98	1.21	0.68
	Ammonium sulfate fraction (35%)	1.30	0.00	0.34	0.00
	Ammonium sulfate fraction (65%)	16.52	8.06	3.56	2.82

* Specific activity=μM of hydroxamate hydrolysed/hr./mg. protein

The supernatant fluid was added with solid ammonium sulfate to 35 per cent saturation under cooling. The precipitate formed was centrifuged off, and solid ammonium sulfate was added to 65 per cent saturation to the supernatant fluid. Again, the precipitate formed was centrifuged, dissolved in a small amount of phosphate buffer (pH 6.8) and dialysed overnight against flowing water, followed by dialysis against 0.001 M EDTA solution for 3 hours. By these operations, the specific activity was raised about 4 fold. (Table II)

RESULTS AND DISCUSSION

Properties of the Enzyme Catalysing the Hydroxamate Formation

Formation of Hydroxamate as a Function of Time—When the reaction mixture was incubated at 38°, the formation of hydroxamate increased as a function of time. Equilibrium was attained in 120 minutes as illustrated in Fig. 1.

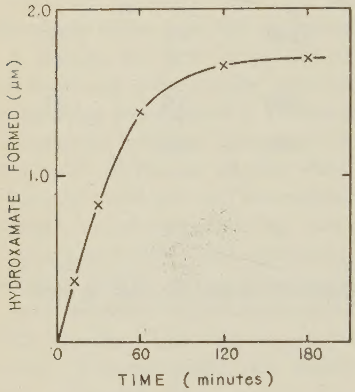

FIG. 1. Hydroxamate formation as a function of time.

Enzyme (Fraction C), 620 μg. of protein; $NaHCO_3$, 40 μM; n-Valerate, 20 μM; NH_2OH, 1000 μM; Total volume, 2.5 ml. 38°.

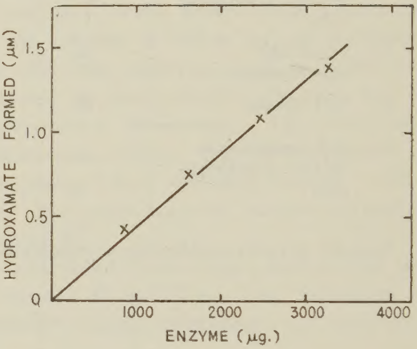

FIG. 2. Rate of Hydroxamate formation as a function of enzyme concentration.

Enzyme (Fraction c) as indicated. $NaHCO_3$, 40 μM; n-Valerate, 20 μM; NH_2OH, 1000 μM; Total volume, 2.5 ml.; 38°, 60 minutes.

Effect of the Enzyme Quantity on Hydroxamate Formation—Fig. 2 gives the relation between the hydroxamate formation and the enzyme quantity. The rate is strictly proportional to the enzyme concentration.

Effect of Substrate Concentration on Hydroxamate Formation—The dependence of the rate of reaction on substrate concentration was determined. From the results illustrated in Fig. 3, the following Michaelis constants were tentatively calculated:

$$K_m = 4.8 \times 10^{-3}\ M \text{ for } n\text{-valerate,}$$
$$K_m = 6.0 \times 10^{-3}\ M \text{ for } n\text{-butyrate.}$$

From similar data obtained in respect of hydroxylamine concentrations, Michaelis constant for hydroxylamine was also tentatively calculated:

$$K_m = 1.2 \times 10^{-1}\ M.$$

Substrate Specificity—Of the acids tested, n-valeric and n-butyric acids served as substrate for the purified enzyme; however, formic, acetic, propionic, caproic, pelargonic, and iso-valeric acids were inert.

Effect of Inhibitors on Hydroxamate Formation—This enzyme was strongly inhibited by some of the so-called sulfhydryl reagents, in particular, by mercuric ion and PCMB. The inhibition by sulfhydryl reagents was reversed

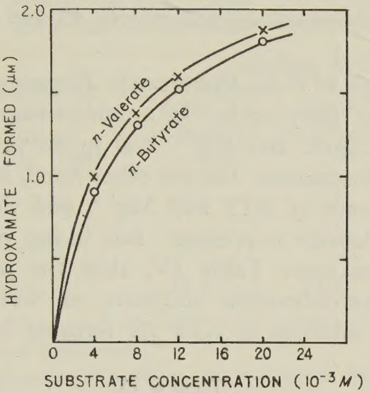

FIG. 3. Effect of substrate concentration on hydroxamate formation.

Enzyme (Fraction C) 620 μg. of protein; $NaHCO_3$, 40 μM; *n*-Valerate (Curve 1) or *n*-butyrate (Curve 2) as indicated; Total volume, 2.5 ml.; 38°, 60 minutes.

TABLE III

Inhibition by Sulfhydryl Reagents for Valerohydroxamate Formation

Inhibitor	(*M*)	L-Cysteine (*M*)	Hydroxamate formed (μM)	Inhibition (%)
None	—	—	1.12	—
$HgCl_2$	1×10^{-3}	—	0.25	78
,,	1×10^{-3}	1.2×10^{-2}	1.28	0
PCMB	8×10^{-4}	—	0.34	70
,,	8×10^{-4}	4×10^{-3}	1.18	0
None	—	—	1.05	—
PCMB	0.8×10^{-4}	—	0.98	7
,,	1.6×10^{-4}	—	0.78	26
,,	3.2×10^{-4}	—	0.68	35
,,	6.4×10^{-4}	—	0.35	67
MIA	4×10^{-4}	—	1.08	0
,,	3.2×10^{-3}	—	1.38	0

Enzyme (Fraction C) 620 μg. of protein; $NaHCO_3$, 40 μM; *n*-Valerate, 20 μM; NH_2OH, 1000 μM; Inhibitors and L-cysteine as indicated; 30°, 60 minutes.

by cysteine as shown in Table II. MIA failed to inhibit the enzyme when added to a final concentration of 10^{-3} *M*. The chelating agents, such as EDTA and Nitroso-R salt were without effect.

Heat Inactivation—When the purified enzyme was heated for 10 minutes at 50° and 60°, the enzyme lost approximately 49 and 95 per cent respectively of its original activity.

Non-participation of ATP in Hydroxamate Formation—There are two kinds of activation process of fatty acids. In animal tissues, acyl-CoA is formed in the presence of ATP, CoA, and Mg^{++}, and in the presence of hydroxylamine, it is converted to hydroxamate. On the other hand, in bacteria acyl-phosphate is formed in the presence of ATP and Mg^{++}, and then gives rise to hydroxamate when hydroxylamine is present. But, in the present study it has been known, as can be seen from Table IV, that the purified enzyme from *M. avium* grown in glycerol-bouillon medium, as well as the crude extract therefrom, needed no addition of ATP for forming hydroxamate.

TABLE IV

Effect of ATP for Valerohydroxamate Formation

Addition	Hydroxamate formed (μM)
Complete system	1.75
No ATP	1.80
No substrate	0
No enzyme	0

Complete system contains in 2.5 ml. : Enzyme (Fraction F), 128 μg. of protein ; Tris buffer (pH 8.0) 25 μM ; *n*-valeratc, 20 μM NH_2OH, 1000 μM ; ATP, 2.5 μM ; 38°, 60 minutes.

Comparison with Esterase—Lipmann and Tuttle (*6*) reported that the hydroxamate was formed by the action of lipase directly from fatty acid and hydroxylamine of high concentration. Therefore it is necessary to compare our enzyme with lipase. Since *M. avium* (strain Takeo) contains heat-stable esterase instead of lipase as reported by Yamamura *et al.* (*7*), the properties of esterase were compared with those of the enzyme under consideration. As shown in Table V, the purified enzyme (Fraction F) exhibited no esterase activity toward tributyrin at all. It is evident, therefore, that the hydroxamate formation in our system is not due to the presence of esterase of broad specificity.

In the crude enzyme preparation esterase is contained, however, the hydroxamate forming activity of the preparation behaved differently from the esterase activity in regard to the heat inactivation. Namely, the esterase withstood heating at 60° for 10 minutes, while the hydroxamate forming enzyme was completely inactivated as shown in Table VI.

TABLE V

Test of Esterase Activity in Purified Enzyme Preparation Catalysing Valerohydroxamate Formation

Experiment No.	Valerohydroxamate formed (μM)	Esterase activity Ammounts of tributyrin		
		Before incubation (μM)	After incubation (μM)	Enzymatic hydrolysis (μM)
I	0.52	5.0	5.3	0.0
II	1.09	8.2	8.5	0.0
III	1.10	18.1	18.4	0.0

For hydroxamate formation :
Enzyme (Fraction F), 32 μg. of protein in Experiment 1 and 64 μg. of protein in Experiment II and III ; Tris buffer (pH 8.0), 30 μM ; *n*-Valerate, 20 μM ; NH_2OH, 1000 μM ; 38°, 120 minutes.

For hydrolysis of tributyrin :
Enzyme (Fraction F), 32 μg. in Experiment I and 64 μg. in Experiment II and III ; Tris buffer (pH 8.0), 30 μM ; Tributyrin as indicated ; Esterase activity was measured by the method of Stern *et al.* (*5*) ; 38°, 120 minutes.

TABLE VI

Heat Stability of Esterase and the Enzyme Catalysing the Hydroxamate Formation

Heat treatment	Valerohydroxamate Formation		Esterase activity	
	Hydroxamate formed	Inhibition per cent	Hydrolysis of ethyl valerate	Inhibition per cent
Intact	6.75 (μM)	—	6.7 (μM)	—
60°, 10 min.	0.08	99	4.0	37
80°, 10 min.	0.10	99	4.0	37

For hydroxamate formation
Enzyme (Fraction B) 0.5 ml. ; *n*-Valerate, 100 μM ; NH_2OH, 1000 μM.

For hydrolysis of ethyl Valerate
Enzyme (Fraction B) 0.5 ml. ; Ethyl valerate, 100 μM : Esterase activity was measured by the method of alkaline titration (*4*). 38°, 120 minutes.

In addition, esterase is distinguished from hydroxamate forming enzyme in being more sensitive to PCMB and Hg^{++} than the latter, as indicated in Table VII.

TABLE VII

Inhibition of Eeterase and the Enzyme Catalysing the Hydroxamate Formation

Experiment No.	Inhibitor	Valerohydroxamate Formation		Esterase activity	
		Hydroxamate formed	Inhibition per cent	Hydrolysis of tributyrin	Inhibition per cent
I	None	1.20 (μM)	—	2.40 (μM)	—
	PCMB, $1.3\times10^{-4}\,M$	0.94	22	1.51	37
	$HgCl_2$, $2.6\times10^{-4}\,M$	1.08	10	0.06	97
II	None	4.58	—	2.13	—
	$HgCl_2$, $1.5\times10^{-4}\,M$	4.34	5	0.95	56

Experiment I:

For hydroxamate formation:

Enzyme (Fraction C), 0.1 ml; Tris buffer (pH 8.0), 15 μM; *n*-Valerate, 20 μM; NH_2OH, 1000 μM;

For hydrolysis of tributyrin:

Enzyme (Fraction C), 0.1 ml.; Tris buffer (pH 8.0), 15 μM; tributyrin, 7.9 μM; If added, PCMB, $HgCl_2$ as indicated. Esterase activity was measured by the methed of Stern *et al.* (*9*). 38°, 60 minutes

Experiment II:

For hydroxamate formation:

Enzyme (Fraction C), 0.5 ml.; *n*-Valerate, 100 μM; NH_2OH, 1000 μM;

For hydrolysis of tributyrin:

Enzyme (Fraction C), 0.5 ml.; Tributyrin, 20 μM; If added, $HgCl_2$ as indicated. Esterase activity was measured by the method of alkaline titration (*4*). 38°, 60 minutes.

Properties of the Enzyme Catalysing the Hydroxamate Hydrolysis

Hydrolysis of Hydroxamate as a Function of Time—The hydrolysis of the hydroxamates was investigated as a function of time:

VHA was hydrolysed to 37 per cent, BuHA to 31 per cent and BenzHA to 47 per cent, while NHA was hydrolysed to 97 per cent at 38° for 60 minutes. Results are illustrated in Fig. 4.

Effect of Substrate Concentration on Hydroxamate Hydrolysis—Fig. 5 shows the relation between the concentration of NHA, BenzHA, VHA, and BuHA and the rate of hydroxamate hydrolysis. From the data obtained, Michaelis constants were tentatively calculated for each substrate:

$K_m=1\times10^{-3}\,M$ for NHA,
$K_m=2\times10^{-4}\,M$ for VHA,
$K_m=5\times10^{-4}\,M$ for BuHA.

Effect of pH on Hydroxamate Hydrolysis—The optimum pH of the enzyme action was determined with NHA and BenzHA as substrate. It was found

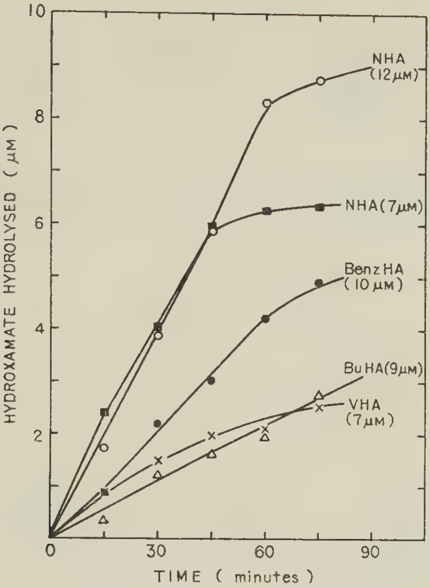

FIG. 4. Hydroxamate hydrolysis as a function of time.

Enzyme (Ammonium Sulfate 65 per cent Fraction), 573 μg. of protein; Phosphate buffer (pH 7.3), 200 μM; Substrate, as indicated, Total volume, 4.0 ml. 38°.

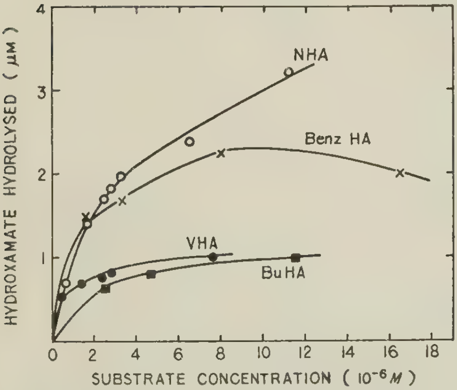

FIG. 5. Effect of substrate concentration on hydrolysis of hydroxamate.

Enzyme (Ammonium sulfate 65 per cent fraction), 220 μg. of protein; Phosphate buffer (pH 6.8), 200 μM; Substrate as indicated; Total volume, 4.0 ml.; 38°, 60 minutes.

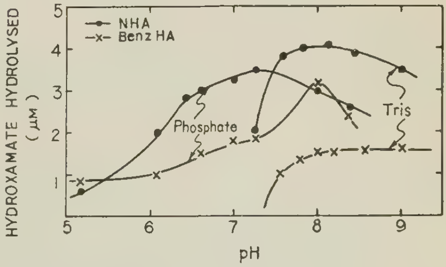

FIG. 6. Effect of pH on hydrolysis of hydroxamate.

Enzyme (Ammonium sulfate 65 per cent fraction), 220 μg. of protein; Phosphate or tris buffer, 200 μM; Nicotinohydroxamate, 6 μM; Benzohydroxamate, 10 μM; Total volume, 4.0 ml. 38°, 60 minutes.

for the hydrolysis of NHA 7.3 with phosphate buffer and 7.8 with Tris buffer, and for that of BenzHA 8.0 with phosphate buffer. (Fig. 6)

TABLE VIII-a

Effect of Metal Ions on Hydrolysis of Hydroxamate

Substrate Metal (*M*)		Nicotino-hydroxamate (12 μM)	Benzo-hydroxamate (10 μM)
None		5.48	2.92
Co^{++},	5×10^{-4}	5.70	2.84
Ba^{++},	5×10^{-4}	6.22	2.72
Ca^{++},	5×10^{-4}	6.22	2.82
Ni^{++},	5×10^{-4}	5.74	2.42
Mn^{++},	5×10^{-4}	6.12	2.74

The values show the amounts of hydroxamate hydrolysed. Enzyme (Ammonium sulfate 65 per cent fraction), 382 μg. of protein; Phosphate buffer (pH 7.3), 200 μM; Substrates and metal ions as indicated; Total volume, 4.0 ml. 38°, 60 minutes.

TABLE VIII-b

Effect of Metal Ions on Hydrolysis of Hydroxamates

Substrate Metal (*M*)		Nicotino-hydroxamate (12 μM)	Benzo-hydroxamate (8 μM)	*n*-Valero-hydroxamate (5.5 μM)	*n*-Butyro hydroxamate (8 μM)
None		4.88	2.46	1.44	1.20
Hg^{++},	1.3×10^{-4}	0.00	0.21	0.00	0.00
Cu^{++},	$5\ \times10^{-4}$	5.12	1.80	1.00	0.76
Zn^{++},	,,	5.74	2.40	1.14	1.02
Mg^{++},	,,	5.83	2.64	1.22	1.08
Cr^{+++},	,,	6.58	2.82	1.38	1.38

The values show the amounts of hydroxamate hydrolysed.

Enzyme (Ammonium sulfate 65 per cent fraction), 382 μg. of protein; Phosphate buffer (pH 7.3), 200 μM; Substrates and metal ions as indicated; Total volume, 4.0 ml. 38°, 60 minutes.

Effect of Metal Ions on Hydroxamate Hydrolysis—Table VIII-a,b shows the effect of metal ions on the enzymatic hydrolysis of hydroxamate. With NHA, BenzHA, and BuHA as substrate respectively, the reaction was greatly inhibited by Hg^{++}, but not by Cu^{++}, Zn^{++}, or Mg^{++}. Cr^{+++} slightly raised the activity, and Co^{++}, Ba^{++}, Ni^{++}, and Mn^{++} had little effect on the hydrolysis of NHA, and BenzHA.

Heat Inactivation—The enzyme catalysing the hydrolysis of nicotinohydroxamate was highly unstable to heat: it was completely inactivated by heating at 50° for 5 minutes.

Effect of Inhibitors on Hydroxamate Hydrolysis—The effect of metal catchers on the purified enzyme was investigated. α,α'-Dipyridyl, *o*-phenanthroline, EDTA, and nitroso-R salt exerted no significant inhibition, as shown in Table IX.

TABLE IX

Effect of Inhibitors on Hydrolysis of Nicotinohydroxamate

Inhibitor	(*M*)	Hydroxamate hydrolysed
None		5.48
α,α'-Dipyridyl	5×10^{-5}	3.44
EDTA	1.3×10^{-3}	3.64
Nitroso-R	2.5×10^{-4}	3.22
o-Phenathroline	1.3×10^{-4}	3.38
NaRSO	1.3×10^{-3}	3.46
KCN	2.5×10^{-4}	3.40
NH_2OH	1.3×10^{-3}	2.62

Enzyme (Ammonium sulfate 65 per cent fraction), 382 μg. of protein; Phosphate buffer (pH 7.3), 200 μM; Nicotinohydroxamate, 12 μM; Total volume, 4.0 ml. 38°, 60 minutes.

Specificity—From the results obtained, it remains as yet uncertain whether the formation and hydrolysis of hydroxamate are catalysed by two different enzymes, though these enzymes have some differences in respect of heat stability and substrate specificity.

The enzyme catalysing the nicotinohydroxamate hydrolysis and nicotinamidase are different not only in their behavior toward heavy metal inhibitors but also in their optimum pH. In addition, nicotinohydroxamate was not hydrolysed at all by *M. phlei* though nicotinamide was hydrolysed. In this regard the findings of Grossowicz *et al.* (*8*) are worthy of note that asparaginase was to be distinguished from hydrolase of aspartohydroxamate with respect to the stereo-specificity of substrates. Therefore it seems likely that the enzyme catalysing the nicotinohydroxamate hydrolysis of *M. avium* is also distinguished from nicotinamidase.

SUMMARY

1. The formation of hydroxamate from *n*-valeric and *n*-butyric acid and hydroxylamine, and the hydrolysis of nicotinohydroxamate, benzohydroxamate, *n*-valerohydroxamate, and *n*-butyrohydroxamate are demonstrated with the purified enzyme preparation obtained from *Mycobacterium avium* (strain Takeo).

2. The enzyme for hydroxamate formation was purified about 19 fold and that for hydroxamate hydrolysis about 4 fold.

3. The purified preparations catalysing the formation of hydroxamate are completely free of esterase activity, and even in the crude preparations the properties of both enzymes can be sharply distinguished. Hence the hydrolysis of hydroxamate and that of amide might be catalysed by two different enzymes.

It is a pleasure for the author to express his gratitude to Prof. S. Akabori of the University of Osaka for his continuous guidance throughout this series of the studies. His thanks are also due to Prof. T. Sasakawa for his kind help and interest and to Messrs. K. Suzuki and S. Satoh for their technical assistance.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

TRYPSINOGEN-KINASE IN ASPERGILLUS ORYZAE

IV. A STUDY OF ACID-PROTEASE PROPERTIES

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(Received for publication, January 9, 1959)

It was previously reported (*1*) that an enzyme found in *Asp. oryzae* and Takadiastase activated trypsinogen to trypsin and the purified sample of this enzyme was homogeneous by paper electrophoresis. It was further stated that the acid-protease and trypsinogen-kinase could be considered as identical substances since the ratio between their activities appeared to remaine approximately constant throughout the various stages of the purification process.

According to Miura (*2*) and Crewther (*3*), at least three different proteases acting in alkaline, neutral or acidic media are present in *Asp. oryzae* or Takadiastase. However, no detailed study of the specific properties of these proteases has been carried out except for the investigation by Sanger (*4*) who observed decomposition of insulin with Crewther's crystalline protease. This enzyme is presumably an alkaline-protease. The proteolytic processes by these enzymes are obviously completely different from one another and it is quite difficult to determine the relative potency of each enzyme. Moreover, the absolute as well as relative amounts of these proteases differ depending upon species and cultural methods (*5*) (*6*). In general, however, the principal protease in *Asp. oryzae* is alkaline-protease.

Acid-protease, which is contained in the least amount and apparently possesses the weakest proteolytic activity, exhibits trypsinogen-kinase activity. Furthermore, according to Isojima (*7*), it despecializes horse serum. These facts are of interest since no other proteases exhibit such specific activity. Although no experimental datum was obtained sufficient to explain this specific activity of the acid-protease, some properties of this enzyme were studied in this paper.

EXPERIMENTALS

Materials and Methods—Purified samples of acid-protease were obtained by the method described previously. Trypsinogen, pepsin and soybean trypsin inhibitor employed in this study were all G. B. I. crystalline preparations. The various peptides used were synthesized at Akabori Laboratory of the Faculty of Science, Osaka University. The measurements of acid-protease and trypsin were made by the previously-described methods and pepsin was measured in accordance with the method applied to determine acid-protease. Ninhydrin color reactions were conducted by the Moore and Stein method (*8*).

Experiment I. Activation Rate of Trypsinogen and Amount of Acid-Protease

Fig. 1 shows the relationship between the activation rate of trypsinogen and amount of acid-protease. Maximum activation occurred with *ca.* 15 μg./ml. of the enzyme.

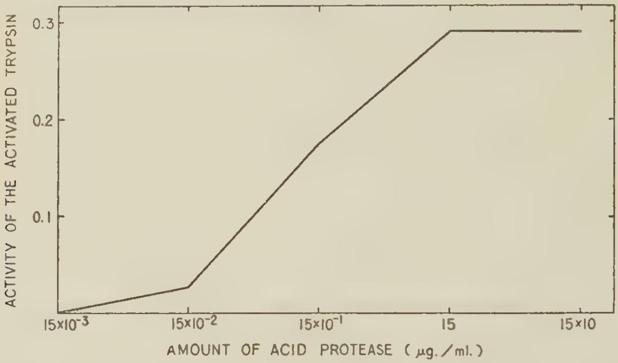

FIG. 1. Activation rate of trypsinogen and amount of the acid-protease. Trypsinogen solution, 2 mg./ml., was mixed with an equal volume of the acid-protease solution of various concentrations and was activated at 0° for one hour. Then the amount of activated trypsin was measured.

Experiment II. Activation Rate of Trypsinogen and Time of Activation

Under the same conditions as shown in Experiment I, the activation rates were measured in various activation times. Maximum activation was found to occur in about 4 hours (Fig. 2).

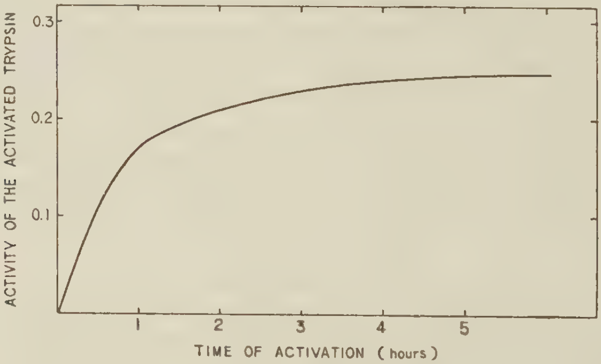

FIG. 2. Activation rate of trypsinogen and time of activation. The experiment was carried out under the same conditions as those described in Fig. 1. 3 μg./ml. of the acid-protease was used.

Neurath (*9*) reported that trypsinogen was activated by *ca.* 80 per cent when treated for 8 hours with 0.5 per cent amount of trypsin and Yamashina (*10*) observed ca. 50 per cent activation in 8 minutes with 15 per cent amount of purified enterokinase. In the present study it was revealed that 100 per cent activation was observed in 4 hours by the use of 0.5 per cent amount of the acid-protease. Thus, it followed that the maximum activation was resulted from the use of trypsinogen-kinase obtained from *Asp. oryzae*. As previously reported by the author, amount of the enzyme sufficient to determine trypsinogen-kinase activity was only one hundredth as compared with that required to determine acid-protease activity. This fact suggests that activation by trypsinogen-kinase takes place as the result of slight proteolysis.

Experiment III. Hydrolysis of Synthetic Peptides

Synthetic substrates listed in Table 1 were used in this experiment.

TABLE I

Hydrolysis of Synthetic Substrates by Acid-Protease

Substrates	Final concentration ($10^{-3}M$)	Hydrolysis (%)
Acetyl-Gly	2	0
Acetyl-DL-Phenylala	2	4
Cl-Acetyl-DL-Met	2	7
Cl-Acetyl-DL-Ser	2	2
Cl-Acetyl-L-Leu	2	23
Gly-Gly-L-Leu	1	0
Gly-L-Leu	2	0
L-Leu-L-Ala	2	4
Gly-L-Asp	2	0
Gly-Gly	2	0
D-Ala-L-Ala	2	0
Gly-L-Ala	2	0
L-Leu-Gly	2	2
Cbz-DL-Ala-Gly-L-Leu	1	69

Walpole buffer solution pH 3.5, 0.5 ml., the substrate, 0.2 ml., and the acid-protease 200 μg./ml., 0.3 ml., were mixed. After allowing to stand at 30° for 24 hours degree of hydrolysis was determined by ninhydrin color reaction.

Isojima (*7*) reported that Cl-acetyl-Leu, Cl-acetyl-Ala and hippuryl-Leu were hydrolysed while hippuryl-Gly and acetyl-Met were not. The author has found that Cl-acetyl-Leu and Cbz-DL-Ala-Gly-Leu were easily hydrolysed, but other acetyl-amino-acids and peptides were either rarely or not at all affected. Since the so-called acylase in *Asp. oryzae* easily hydrolyses acetyl-Met and Cl-acetyl-Ser, this purified acid-protease cannot be acylase. It seems to have no di- or tri-peptidase activities because free di- or tri-peptides were not hydrolysed.

Experiment IV. Comparison of Acid Protease and Pepsin

To compare the acid-protease with pepsin, casein hydrolysis was performed with the acid-protease at pH. 3.0 and with pepsin at pH 2.0. At suitable intervals, 5 per cent T.C.A.-soluble fractions of casein was tested by Folin and ninhydrin reagents and compared. By the author's method, the specific activities of both the acid-protease and pepsin were shown to be approximately the same. Therefore, the same amounts of the two samples were used in this experiment. As can be seen in Fig. 3, greater hydrolysis rate was obtained with pepsin when determined by the Folin reagent, whereas, when measured by the ninhydrin procedure, the hydrolysis by the acid protease was still continued after cessation of hydrolysis by pepsin. It would appear from these observations that, as compared with pepsin, the activity of the acid-protease was weak in hydrolysing casein into large fragments, while strong in separat-

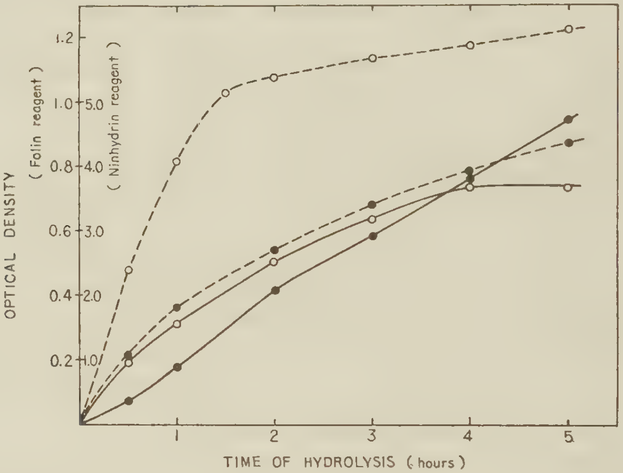

FIG. 3. Comparison of the acid protease and pepsin. Experiments were carried out at pH 3.0 with the acid-protease and at pH 2.0 with pepsin. Sörensen buffer solution (HCl-citrate) pH 2.0 or pH 3.0, 5 ml., 2 per cent casein (HCl) pH 2.0 or pH 3.0, 15 ml., and the enzyme solution (30 μg./ml.), 10 ml. were mixed. After 0.5, 1, 1.5, 2, 3, 4, 5 hours, 3 ml. portions of the reaction mixture were mixed with 3 ml. of 10 per cent T. C. A. Filtrates were tested by the Folin and ninhydrin color reactions.

--○-- Pepsin by Folin, --●-- Acid protease by Folin,
—○— Pepsin by ninhydrin, —●— Acid protease by ninhydrin.

ing it into small fragments. Yoshida (*11*) has obtained similar results in his studies on the acid-protease from *Asp. niger.*

Experiment V. Influence of EDTA on the Acid Protease

Isojima (*7*) reported that activity of his purified enzyme was inhibited by ethylenediamine tetra acetic acid (EDTA) and restored by Zn^{++} and Co^{++}. Yoshida (*11*) observed that EDTA had no inhibitory action on his crystalline enzyme. The casein hydrolysis activity of the author's enzyme was not at all inhibited at pH 3.0 by $10^{-2}\,M$ EDTA as shown in Table II. Since the metal chelating action of EDTA became to be weak at pH 3.0, experiments were planned at pH 4.0. However, these experiments could not be performed, as casein was coagulated under this pH. Subsequent experiments were therefore carried out with Cl-acetyl-L-Leu. But any inhibitory effect was not observed in this case.

TABLE II

Influence of EDTA to Acid-Protease

		EDTA	
Substrate	pH	No addition	Addition (final concentration)
Casein[1)]	3	0.099	0.099 ($10^{-2}\,M$)
Cl-Acetyl-Lau[2)]	4	0.232	0.224 ($2\times10^{-2}\,M$)

1) 2 per cent casein (HCl) pH 3.0, 1.5 ml., Walpole buffer solution, pH 3.0, 0.5 ml., and the acid-protease 30 μg./ml. with or without EDTA, 1 ml., were mixed. After mixing those materials the procedure was the same as previously reported.

2) Cl-acetyl-Leu $10^{-2}\,M$, 0.3 ml., Walpole buffer solution, pH 4.0, 0.5 ml., and the acid-protease 100 μg./ml. with or without EDTA, 0.6 ml., were mixed. The reaction time was 2 hours and the temperature was 35°. All the values are presented in terms of optical density.

Experiment VI. Relation of Soybean Trypsin Inhibitor to Acid-Protease

Matsushima (*12*) recently studied on inhibitors of proteases from *Asp. oryzae.* Miura (*13*) discovered that the acid protease could decompose the inhibitor of the alkaline-protease from *Asp. oryzae* which was found in the white of eggs. In the present study experiments were undertaken to examine the relation of crystalline soybean trypsin inhibitor to acid-protease. It was observed that with equal amounts of the inhibitor and trypsin maximum inhibition was attained. This observation was in agreement with Laskowski's results (*14*). (Fig. 4.)

The inhibitor was used in amount almost 4 times as much as that of acid protease in the study of its effects on the acid-protease, but any inhibitory action was not observed. To study whether the inhibitor was decomposed by the acid-protease or not, the acid-protease was caused to act on the inhibitor for 24 hours, but no loss of the inhibitor activity was observed. These facts suggest that the soybean trypsin inhibitor and acid-protease are completely unrelated to each other. (Table III)

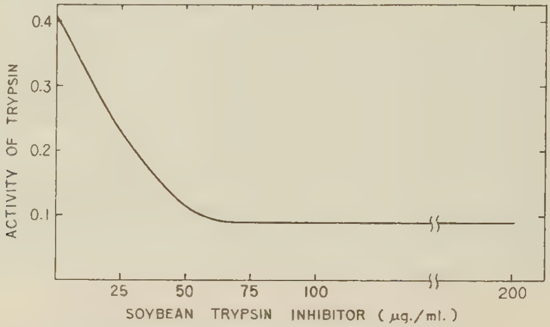

FIG. 4. Inhibition of trypsin by soybean trypsin inhibitor. Trypsin 66 μg./ml., 0.5 ml., the inhibitor solution 0.5 ml., and buffer solution, pH 7.0, 1.0 ml. were mixed. After preincubation for 10 minutes, casein solution (2 per cent, pH 7.0) 1 ml. was added and then trypsin activity was measured.

TABLE III

Relation of Acid Protease to Soybean Trypsin Inhibitor

(a) Inhibition of the acid protease by soybean trypsin inhibitor

	Inhibitor 160 μg.	
Acid protease	No addition	Addition
40 μg.	0.088	0.089

The acid protease was measured as previously reported.

(b) Decomposition of soybean trypsin inhibitor by acid protease

Trysin only 33 μg.	Inhibitor 25 μg. was added.	Inhibitor* 25 μg. which was treated by acid protease was added
0.369	0.118	0.129

* for 24 hours at 30°, pH 3.5

Experiments were carried out as shown in Fig. 4.

SUMMARY

Properties of trypsinogen-kinase from *Asp. oryzae*, which proved to be identical with acid protease, were investigated.

1. Maximum activation of trypsinogen was attained in 4 hours at 0° using 3 μg. of this enzyme to 2 mg. of trypsinogen.
2. Cl-acetyl-Leu and Cbz-DL-Ala-Gly-Leu are hydrolysed by this enzyme.
3. The activity is weak in hydrolysing casein into large fragments, while strong in separating it into small fragments as compared with pepsin.
4. It is not inhibited by EDTA, but a study will be necessary on this point.
5. The acid protease is not inhibited by soybean trypsin inhibitor and cannot decompose this inhibitor.

I wish to express my thanks to Prof. Akabori of Osaka University for his kind guidance and to the staff of the Akabori Laboratory for supplying the peptides used in this study.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

STUDIES ON NITRATE REDUCTASE SYSTEM OF ESCHERICHIA COLI

II. SOLUBLE NITRATE REDUCTASE SYSTEM OF AEROBICALLY GROWN CELLS IN A SYNTHETIC MEDIUM*, **

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Nitrate reductase system from *Escherichia coli* Yamagutchi strain, grown aerobically on a complex peptone-broth agar medium containing 0.1 per cent KNO_3, has been recently characterized by Iida *et al.* (*2*) as a particulate system containing cyt. b_1*** as an indispensable electron carrier. By the isobutanol or deoxycholate treatment, the particulate system could be solubilized being accompanied by a simultaneous blocking of the linkages connecting formate and DPNH with cyt. b_1.

On the other hand, Nicholas and Nason (*3*) have reported a soluble DPNH linked NaR of molybdoflavoprotein nature requiring no cytochrome in *E. coli* strain B which had been grown aerobically in a synthetic medium presumably by assimilating nitrate.

When our strain of *E. coli* was cultured in the same medium except that a small amount of yeast extract was added to the medium, the nitrate in the medium was reduced only to the nitrite stage, indicating the non-assimilative nature of the reduction. The cultured cells possessed an active NaR comparable to that of complex medium cells. It seemed noteworthy, however, that a considerably increased amount of soluble form of NaR could be easily extracted from the cells grown on a synthetic medium.

The present paper deals with the purification and properties of this soluble NaR system. Electron transport system leading to nitrate in the soluble extract, its purified preparation, and the particulate fraction from the same organism were investigated and compared.

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* A part of this study was already presented at the "International Symposium on Enzyme Chemistry", held in Tokyo in 1957 (*1*).

** This work was supported by a grant from the Scientific Research Fund of the Ministry of Education of Japan.

*** Following abbreviations are used: cytochrome, cyt.; nitrate reductase, NaR; phenosafranine and its reduced form, Ps and PsH_2; methylviologen and its reduced form, MV and MVH; diphosphopyridine nucleotide and its reduced form, DPN and DPNH; flavin adenine dinucleotide and flavin mononucleotide, FAD and FMN; formic dehydrogenase, FDH.

MATERIALS AND METHODS

Microorganism—Escherichia coli Yamagutchi strain was grown in a medium consisting of the following: per liter*; Na_2HPO_4, 6 g.; KH_2PO_4, 3 g.; $MgSO_4$ $7H_2O$, 0.2 g.; NaCl, 0.5 g.; NH_4Cl, 0.1 g.; $NaNO_3$, 1 g.; glucose, 4 g.; yeast extract powder, 0.5 g. One liter of the medium in 5 liter Erlenmyer flask was inoculated and kept on a shaking machine at 30° for 18 hours. The cells were harvested by a Sharples Ultracentrifuge and were washed with cold distilled water to remove nitrite.

Other Materials—The calcium phosphate gel used in the purification procedure was prepared by the method described by Keilin and Hartree (*4*). DPNH and TPNH were prepared by enzymic reduction by the methods described by Pullman *et al.* (*5*) and by Nason *et al.* (*6*), respectively. FAD and FMN of about 80 per cent purity was kindly donated by Dr. K. Yagi.

Preparation of Crude Extracts, Soluble Extracts and Particulate Fractions—All procedures were carried out at 0–4°. The cell-free crude extracts were prepared by grinding the packed cells in frozen state in a cold mortar for 40 minutes with 1.5 times their weight of alumina powder (Wako No. 800) and for further 20 minutes with 3 times their weight of cold 0.1 *M* phosphate buffer, pH 6.8 containing 10^{-3} *M* EDTA. Large masses of unbroken cells, debris and alumina powder were removed by centrifugation at $2{,}000 \times g$, for 20 minutes to yield the cell-free extract designated as $S_{0.2}$. The sedimented materials were extracted again as before and the extracts were combined. By the centrifugal fractionation at $20{,}000 \times g$, for 40 minutes (International centrifuge) and $107{,}000 \times g$, for 1 hour (Preparative Spinco model L) of $S_{0.2}$, four fractions designated as $S_{2.0}$**, $P_{0.2-2.0}$, $S_{10.7}$ (these three are, in the following, called the crude extract, the particulate fraction and the soluble extract, respectively.) and $P_{2.0-10.7}$ were obtained.

Enzyme Assay—All assays were carried out anaerobically in the Thunberg tube at 30°, in a manner similar to that previously described (*2*). After 10-20 minutes of reaction, a suitable amount of neutralized $CdSO_4$ solution was added and with the deproteinized clear supernatant solution the amount of nitrite produced was measured colorimetrically in the usual manner (*7*)***.

NaR—This activity was assayed by using PsH_2 (prepared by reduction of Ps with palladium and H_2 gas) as an electron donor. The reaction mixture contained 0.05 *M* citrate phosphate buffer, pH 6.4, 2.5 μmoles of PsH_2, 5 μmoles of KNO_3 and enzyme preparation in a final volume of 2.5 ml. 5 μmoles of MVH (reduced with dithionite) was sometimes used in place of PsH_2. In the present paper, the term "NaR" only refers to the electron-transferring activity from PsH_2 to the nitrate assayed as above.

Formate-NaR—This activity was assayed by using formate as a hydrogen donor and the working enzyme system was thus termed. The reaction mixture was the same as above except that PsH_2 was replaced by 25 μmoles of sodium formate.

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* The addition of the yeast extract enhanced not only the total growth but the total units and the specific activity of NaR of the cells.

** $S_{2.0}$ is the supernatant obtained at $20{,}000 \times g$. $P_{0.2-2.0}$ and the like refer to the particulate fractions obtained between 2,000 and $20{,}000 \times g$, *etc.*

*** Since DPNH interferes with the development of azo dye in the determination of nitrite produced in DPNH-NaR reaction, the reaction mixture, prior to the addition of $CdSO_4$ solution, was vigorously shaken and left to stand in an open tube at 30° for about 5 minutes to oxidize remaining DPNH by means of DPNH oxidase in the soluble extract used or externally added.

DPNH-NaR—This activity was assayed by using DPNH as a hydrogen donor and the working enzyme system was thus termed. The reaction mixture contained 0.05 M citrate phosphate buffer, pH 6.4, 0.5 μ moles of DPNH, 3 μ moles of KNO_3 and the enzyme preparation in a final volume of 3.0 ml. . The decrease of optical density at 340 $m\mu$ was followed by a Hitachi spectrophotometer against the reference open cuvette without DPNH.

Assay of FDH activity and the definition for the enzyme unit and specific activity are the same as those previously described (*2*).

RESULTS

Non-Assimilative Nitrate Reduction and NaR Activity of the Cells—During the cultivation, nitrite produced from nitrate accumulated in the medium to the level about 60–70 per cent of the initially added nitrate when the maximal growth was attained after 18 hours. In no cases, appreciable activity of nitrite reductase in the harvested cells was detected under varing anaerobic conditions. From these observations, it can be concluded that *E. coli* Yamagutchi strain could not assimilate nitrate-N but ammonia- or organic-N present in the yeast extract.

The cells were found to be capable of reducing nitrate by utilizing various electron donors ; formate, lactate, succinate and the reduced dyes including PsH_2 and MVH.

Distribution of NaR, FDH and Cyt. b_1 *after Centrifugal Fractionation of the Supernatant at* $2{,}000\times g$ $(S_{0.2})$

TABLE I

Distribution of NaR and FDH after Centrifugal Fraction of $S_{0\cdot2}$

Fraction	Total N (*mg.*)	NaR		Formic dehydrogenase	
		Units	Specific activity	Units	Specific activity
Whole cells	26.0	145	5.6	—	—
$S_{0\cdot2}$	16.0	157	9.7	115	7.2
$P_{0\cdot2-2\cdot0}$	6.7	75	11.3	230	34.4
$S_{2\cdot0}$	9.5	87	9.1	0	0.0
$P_{2\cdot0-10\cdot7}$	1.3	21	16.0		
$S_{10\cdot7}$	7.6	65	8.6		

Concerning the conditions, see the "Materials and Methods".

As shown in Table I, about 30–40 per cent of NaR activity of the whole cells could be recovered in the soluble extract. This result profoundly differs from the previous result obtained by employing ultrasonic treatment or alumina-grinding method for complex medium cells (*2*). In the complex medium cells, the presence of considerable amounts of NaR activity in the particulate fraction and only about 5 per cent recovery in the soluble extract were shown. In the present crude extract, no FDH activity was observed, whereas an appreciable amount of this activity was found in the similar fraction from the complex medium cells. Particulate fraction has twice as much total units of FDH as those of the $S_{0.2}$, suggesting the presence in $S_{0.2}$ of some uncharacterized factor inhibiting this enzyme activity. A large amount of cyt. b_1 was found to reside in the particulate fraction though a small portion was demonstrated in the soluble extract as described below (Fig. 3.).

A distribution pattern similar to that recorded in Table 1 was also confirmed by employing the ultrasonic irradiation for disrupting cells. Thus, the differences from the distribution pattern of NaR, FDH and cyt. b_1, which was obtained from the complex medium cells seem to be due not to the difference of the cell-rupturing method but to the different growth conditions. The opposite tendencies of these two enzymes, *i.e.* NaR to localize chiefly in the soluble extract ($S_{10.7}$) and FDH to be strictly bound to the particulate fraction ($P_{0.2-2.0}$), seem to be favorable for further purification of the terminal enzyme, NaR.

In contrast with the soluble systems, only the particulate fraction which was characterized by the higher level of cyt. b_1 and dehydrogenase activity had formate-NaR. Lactate, succinate, DPNH, PsH_2 and MVH were also effective donors.

As seen from Table I, NaR activity of the particulate fraction and that of the soluble extract are equally responsible for the whole activity of the synthetic medium cells.

Further studies confirmed that the present particulate system had quite similar nature to that obtained from the complex medium cells (*2*)*. In the present studies, therefore, the emphasis was laid on the investigation of the soluble systems.

Purification of the Soluble Systems from the Crude Extract and the Removal of Electron-Transferring System from DPNH—The crude extracts were brought to 19 per cent saturation with solid ammonium sulfate and allowed to stand for 20 minutes. After centrifugation at 20,000×*g*, for 10 minutes, the precipitate was discarded. The ammonium sulfate concentration of the supernatant solution was raised to 36 per cent saturation and the mixture was allowed to stand for 20 minutes. The precipitate collected by centrifugation was dissolved in one-fourth volume of the original extract of cold 0.1 M phosphate buffer of pH 6.8 and dialyzed with stirring for two hours against $5\times10^{-3}\,M$ of the same phosphate buffer containing $10^{-4}\,M$ cysteine. The dialyzed preparation was centrifuged to remove the insoluble materials. To the supernatant was added the same volume of calcium phosphate gel (about 30 mg. dry weight per ml.) and stirred for 20 minutes. After centrifugation, the supernatant fluid was discarded and the gel was washed successively twice with cold distilled water, twice with 0.1 *M* phosphate buffer of pH 7.4 of 3 times the volume of added gel solution. One-fourth volume of 0.25 *M* pyrophosphate buffer of pH 7.4 was mixed with the gel and, after standing for 10 minutes, the eluate was collected by centrifugation for 15 minutes. This treatment was repeated 2 to 3 times further. The combined eluates were dialyzed for 2 hours against 0.05 *M* phosphate buffer of pH 6.8 containing 10^{-4} *M* cysteine and used as the purified preparation. The summary of the purification is recorded in Table II.

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* As found previously in the case of complex medium cells (*2*), it was found that the soluble extract also contained heat stable activator for this particulate formate- or DPNH-NaR.

TABLE II

Summary of the Purification of Soluble NaR

Fraction	Total N (*mg.*)	Units of NaR		Specific activity (from PsH_2)	Recovery (*per cent*)
		from PsH_2	from DPNH		
Crude extract ($S_{2.0}$)	3.90	34.0	6.8	8.7	—
19–36 per cent ppt. (Am_2SO_4)	0.96	17.4	—	26.5	51
Calcium phosphate gel eluate	0.10	8.3	0.0	83.0	24

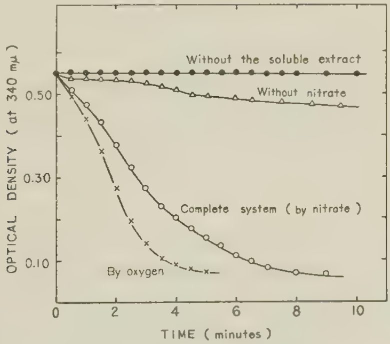

FIG. 1. DPNH oxidation by nitrate or oxygen in the presence of the soluble extract. In the standard assay of DPNH-NaR, 0.12 μ moles DPNH and the soluble extract of 0.5 mg. N were used.

The high speed supernatant (107,000 × *g*, 1 hour) of the purified preparation contained 95–100 per cent of the initial activity. On the other hand, as shown in Table II, DPNH could not serve its electrons for the nitrate reduction by the gel eluate even in the presence of menadione, FAD and

Fe^{++} indicating the elimination during the purification of the system transferring electron from DPNH.

The Soluble System Transporting Electron to Nitrate—Since about 80 per cent of DPNH-NaR activity of $S_{0.2}$ was recovered in its ultracentrifugal supernatant ($S_{10\cdot7}$) in a soluble form, the electron transport system from DPNH to nitrate was studied by using the soluble extract.

(*a*) *The Enzymic Oxidation of DPNH by the Soluble Extract*: The enzymic oxidation of DPNH by nitrate is shown in Fig. 1. The stoichiometric relationship between oxidized DPNH and produced nitrite was established. TPNH was found to be less effective.

Fig. 1 shows about 2-fold more rapid oxidation rate by molecular oxygen than by nitrate, indicating the presence of an active DPNH oxidase system.

(*b*) *Flavin Participation*: When examined fluorometrically by the lumiflavin method* the soluble extract was found to contain an appreciable amount of flavin components. The DPNH-NaR activity of the soluble extract which was slightly activated by the addition of FAD was much enhanced by preliminary dialysis of the extract for 3 hours. As shown in Table III, the dialysis lowered the activity of the soluble DPNH-NaR, while no significant decrease was observed in the activity measured by using PsH_2.

TABLE III

Stepwise Activation of DPNH-NaR in the Soluble Extract

Electron donor	System	Units
DPNH	(1) Soluble extract	0.23
	(2) Dialyzed soluble extract	0.08
	(3) (2)+FAD (10^{-5} *M*)	0.15
	(4) (3)+menadione (10^{-4} *M*)	0.19
	(5) (4)+Fe^{++} (5×10^{-4} *M*)	0.48
PsH_2	(1) Soluble extract	0.95
	(2) Dialyzed soluble extract	0.87

The soluble extract (0.08 mg. N) was used in the standard assay of DPNH-NaR.

(*c*) *Stepwise Activation of DPNH-NaR in the Soluble Extract*: The soluble extract (0.08 mg. N) was used in the standard assay of DPNH-NaR.

Under these conditions, the addition of FAD (FMN was equally effective) resulted in 2- to 2.5-fold activation at 10^{-5} *M*. The effect of FAD and FMN concentration on the soluble DPNH-NaR is shown in Fig. 2.

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* The authors are indebted to Dr. J. Okuda of the Department of Biochemistry, the Medical School of Nagoya University for performing the fluorometric analysis of flavin.

The flavin participation was also supported by inhibitory effect of amytal (*8*) and quinine (*9*) on the DPNH-NaR activity in the undialyzed soluble extract as recorded in Table IV.

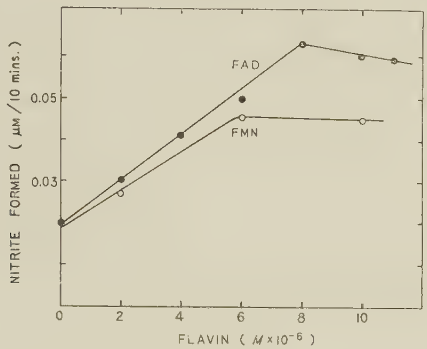

FIG. 2. Flavin nucleotide requirement of DPNH-NaR in the dialyzed soluble extract. The soluble extract (0.3 mg. N) was used in the standard assay. The half maximal activation by FAD occurred at 4×10^{-6} *M*.

(*d*) *Menadione Participation*: Dicumarol and HOQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide)* were the potent inhibitors for the DPNH-NaR activity (Table IV).

Table V shows that the dicumarol inhibition was completely reversed by the addition of 10^{-4} *M* of menadione but not by α-tocopherol. On the contrary, HOQNO inhibition was never overcome by the addition of the same level of these compounds indicating the difference of inhibition site between two inhibitors. Thus, dicumarol, a well-known structural analogue of menadione, may inhibit the menadione reductase which seems to be functioning in the DPNH-NaR of the soluble extract. The further addition of menadione together with FAD to the dialyzed soluble extract resulted in 25–30 per cent activation (Table III).

(*e*) *Cytochrome b_1 Participation*: As shown in Table V, HOQNO seemes to inhibit not menadione reductase but the oxidation site of cyt. b_1. The situation is the same as previously discussed in the case of the particulate system from the complex medium cells (*2*). In fact, a small but the significant amount of cyt. b_1 was spectrophotometrically detected in the soluble extract. The difference spectra of near ultraviolet range between the reduced (with dithionite) and oxidized form of the soluble extract are recorded in Fig. 3. The high level of DPNH or small but a sufficient amount of dithionite was added to reduce the electron-transferring components under anaerobic condition. The difference spectrum obtained by using DPNH was very similar to

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* This was synthesized by Dr. J. W. Cornforth. The sample used in this study was kindly donated by Dr. R. Sato.

TABLE IV

Effect of Inhibitors

Inhibitor	(*M*) (-Log)	Soluble extract	Purified preparation		Particulate fraction
		DPNH	PsH_2	MVH	PsH_2
2,4-Dinitro phenol	3		0		0
NH_2OH	3		15		0
Phenylurethan	3		0	0	0
Amytal	3	40	0	0	0
Quinine	3	30	0	5	0
Dicumarol	3	80	8	0	0
HOQNO[1]	4.5	77	0	0	0
Effect of -SH Inhibitors					
PCMB[3]	3	98 (37)[2]			65
Alloxan	4	65 (0)[2]	20	10	40
	3	100 (20)[2]	47		
Effect of Metal-Binding Inhibitors					
KCN	2	100	90	100	100
	3	75	65	75	79
	4		20		68
	5		0		39
NaN_3	3	100	100	100	80
	4	95	90	90	74
	5	75	47	55	37
CO (dark)	1 atom.	0	0		0
NaF	2		30	20	
α,α'-Dipyridyl	3		30		
8-OH-Quinoline	3	30	0	0	0
Thiourea	2	85	45	46	6
	3		30		0
	4		0		
Allylthiourea	3	30	0	0	12
Salicyl aldoxime	3		0		0
EDTA	2	50	25	15	0

In the assay of NaR of the particulate fraction, phosphate buffer of pH 6.8 was used in place of citrate phosphate buffer of pH 6.4.

1) Ethanol solution was used and simultaneously the control with the same amount of ethanol was run. The half maximal inhibition by this was attained at 3×10^{-7} *M*.

2) Cysteine of 10^{-3} *M* was used.

3) *p*-Chloromercnribenzoate.

TABLE V

Inhibition of DPNH-NaR in the Soluble Extract by Dicumarol, HOQNO and the Effect of D-α-*Tocopherol and Menadione on the Inhibition*

Inhibitor / Further addition	Dicumarol (10^{-4} M)	HOQNO (3×10^{-5} M)
None	80	77
Menadione 2.5×10^{-5} M	29	77
10^{-4} M	0	77
D-α-Tocopherol 20 μg. per ml.	75	70

In the standard assay, menadione in ethanol or D-α-tocopherol in 15 per cent ethanol, 2 per cent bovine serum albumin suspension was used with the appropriate controls.

that reduced by dithionite except that the partial reduction (about 70 per cent) was observed with the former. The positive peak at 430–435 mμ and the negative one at 455 mμ in the difference spectra, being due to the reduction of the Soret peak of cyt. b_1 and flavin respectively, were remarkably decreased by the addition of KNO_3 to the reduced form made by DPNH. A similar but more rapid change was caused by the introduction of air. The

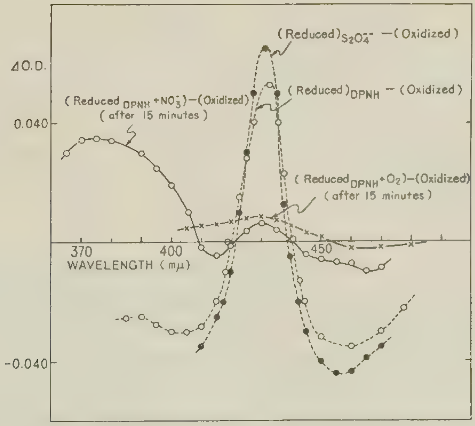

FIG. 3. Difference spectra of the soluble extract (reduced form (with DPNH or $Na_2S_2O_4$) minus oxidized form) and the reoxidation by nitrate or oxygen.
In the standard assay, the soluble extract of 3.8 mg. N (32 units by PsH_2 assay), 3 μ moles of DPNH were used. In the case of the reduction by $Na_2S_2O_4$, about 1.0 mg. thereof was added anaerobically.

change caused by the addition of nitrate agrees with the assumption that the change caused by the addition of KNO_3 and O_2 are due to the reoxidation of reduced cyt. b_1 and flavin. Another peak at about 370–375 mμ occurring after the addition of nitrate seems to be due to nitrite simultaneously produced, though there was a slight discrepancy between the observed peak and the peak of nitrite itself (365 mμ). The simultaneous production of nitrite could also be confirmed by the color test.

The aerobic oxidation of DPNH seems to involve cyt. b_1, since HOQNO (3×10^{-5} *M*) and KCN (10^{-3} *M*) inhibit DPNH oxidation by O_2 (75 per cent) to almost the same extent as that by nitrate.

Small but detectable amount of cyt. b_1 and flavin were found in the purified preparation by spectrophotometric and fluorometric analysis, respectively. The cytochrome content per NaR unit of the purified preparation was about one-fourth that of the soluble extract. Fig. 4 shows the similar difference spectrum of the reduced (with dithionite) and oxidized form in the purified preparation having the similar peak at 430–435 mμ (cyt. b_1) and about 455 mμ (negative and diffused, flavin component). DPNH could never reduce cyt b_1 suggesting the absence of DPNH-cyt. b_1 reductase in the purified preparation. The change of the difference spectrum by the addition of nitrate, though slowly, occurred as before indicating the reoxidation of reduced cyt. b_1 by nitrate. This can be called cyt. b_1-NaR activity. The simultaneous production of nitrite was demonstrated by the color test.

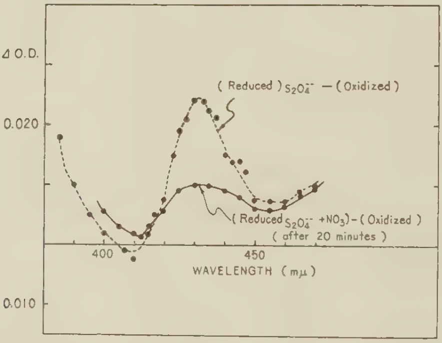

FIG. 4. Difference specra (reduced with $Na_2S_2O_4$ minus oxidized) of the purified preparation and the reoxidation by nitrate.

In the standard DPNH-NaR assay, the purified preparation of 0.4 mg. N (30 units PsH_2 assay) and about 1.0 mg. dithionite in place of DPNH were used.

(*f*) *Ferrous Iron Participation*: As shown in Table IV, all of the reagents capable of binding free metallic ions, as 8-hydroxyquinoline, *o*-phenanthroline, thiourea, allylthiourea and EDTA, were highly inhibitory for DPNH-NaR of the soluble extract. This sensitivity suggests the involvement of some metallic ion in the DPNH-NaR.

Of the metallic ions thus far tested, only Fe^{++} was found to promote DPNH-NaR activity when added to dialyzed soluble extract as shown in Table VI.

TABLE VI

Effect of Metallic Ions

Enzyme preparation	Dialyzed soluble extract	Purified preparation
	Electron donor	
Metallic ion (5×10^{-4} *M*)	DPNH	PsH_2
	Activation per cent	
$FeSO_4$	140	19
$FeCl_3$	45	–5
Na_2MoO_4	10	12
$MoO_3$1)	10	-6
Na_2WO_4	5	-5
$MnCl_2$	–10	–30
$MgCl_2$	15	5

Standard assay system.

1) MoO_3 was dissolved in NaOH at pH about 11 and then acidified with HCl to pH about 4.5 and used.

As shown in Table III, the activity of the dialyzed soluble extract previously activated by FAD and menadione could be further enhanced by the addition of Fe^{++} (2- to 2.5-fold further activation) finally exceeding the original activity of undialyzed soluble extract.

The Fe^{++} ion participation in DPNH-NaR was further confirmed by testing the ability of Fe^{++} as an enzymic electron donor. Among metallic ions (maintained in the reduced form in the presence of dithionite) thus far tested including Fe^{++}, Co^{++}, Cd^{++}, Mg^{++}, Ni^{++}, Zn^{++}, Mn^{++}, MoO_4^{--} and CrO_4^{--}, Fe^{++} (1.7×10^{-3} *M*) was found to serve electron for nitrate reduction in a pyrophosphate buffer as shown in Table VII. The bivalent form of iron is necessary for functioning as an electron donor, since the addition of dithionite was only required when Fe^{+++} was used. This result shows the occurrence of valence change during the functioning of Fe^{++}. The activity of the undialyzed soluble extract measured by using Fe^{++} was about 20–50 per cent of that measured with PsH_2, but comparable to that measured by

TABLE VII

Fe^{++} as Electron Donor for NaR of the Soluble Extract

System (figures indicate nitrite formed in mμmoles per 10 minutes)							
Fe^{++}					Fe^{+++}		PsH_2
Complete	$-S_2O_4^{--}$	$-NO_3^-$	Boiled	$-Fe^{++}$	Complete	$-S_2O_4^{--}$	
43	45	18	0	18	45	13	247

In the standard assay, electron donor and citrate phosphate buffer were replaced by $FeSO_4$ (final 1.7×10^{-3} *M*) and 0.05 *M* pyrophosphate buffer of pH 6.4 respectively. The reduction of the metallic ions was performed by the addition of 1–2 mg. $Na_2S_2O_4$ in the pyrophosphate buffer with the bubbling of H_2 for 2 minutes before and after the addition. Then the Thunberg tube was immediately evacuated.

DPNH in the presence of FAD, menadione and Fe^{++}.

HOQNO at 3×10^{-5} *M* produced 52 per cent inhibition of the enzyme activity of the purified preparation measured with Fe^{++} indicating the possibility of the participation of cyt. b_1 in the electron transport system from Fe^{++} to nitrate. The possibility of complex formation between HOQNO and Fe^{++} can be excluded by the finding that far less concentration of HOQNO than that of Fe^{++} was required for the inhibition.

When measured with PsH_2 as an electron donor, the addition of Fe^{++} caused but little activation of NaR of the purified preparation as contrasted with the system, DPNH-NaR (Table VI).

(*g*) *-SH Group Participation*: The DPNH-NaR activity was markedly inhibited by PCMB (10^{-4} *M*) and alloxan (10^{-3} *M*) (Table. IV) and the inhibition was almost completely reversed by the subsequent incubation with 10^{-3} *M* of cysteine. The activity of purified preparation measured with PsH_2 or MVH was less inhibited.

Thus, -SH group seems to be more essential when DPNH is used as an electron donor.

Properties of Purified Nitrate Reductase—(*a*) *Enzyme Concentration*: The proportionality between the initial rate of nitrate reduction and the enzyme concentration was established within the range of about 3 units under the standard assay conditions.

(*b*) *pH Optimum*: The effect of pH on the enzyme activity was determined by using various buffers as shown in Fig. 5. The pH optimum was about 6.4 in citrate phosphate or pyrophosphate buffer, whereas it was about 6.8 in phosphate or Tris buffer though no sharp pH optimum was obtained. In the citrate phosphate buffer, NaR activity at pH 6.8 was about 1.5–2.0 times higher than that in other buffers. In fact, 1.5–2.5 times activation of NaR activity was observed by the addition of 25 μmoles of citrate to the reaction mixture containing phosphate buffer.

(*c*) *Enzyme Stability*: By adding 10^{-4} *M* cysteine to the purified prepara-

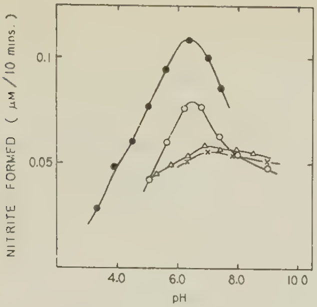

FIG. 5. Effect of pH on NaR activity. In the standard assay, all buffers were used at 0.05 M final concentration. —●—, citrate phosphate; —○—, pyrophosphate; —△—, phosphate; —×—, tris (hydroxymethyl) aminomethane.

TABLE VIII

Reduced Dyes as Electron Donor for NaR

Reduced dyes	Units
None	0.00
Methyl viologen	0.85
Neutral red	0.18
Phenosafranine	0.56
Nile blue	0.48
Methylene blue	0.17
Thionine	0.15
Toluylene blue	0.04
2,6-Dichlorophenol indophenol	0.11

In the standard assay, the reduced dyes prepared by $Na_2S_2O_4$ in the similar manner to that described in the legend of Table VII were used.

tion, NaR can be stored for two weeks in the frozen state (−15°) without a remarkable loss of activity. The enzyme was stable to short dialysis, however, the addition of cysteine or glutathione $(1\sim5\times10^{-4}\,M)$ has been found to be necessary for prolonged dialysis. Heating at 80° for 5 minutes completely destroyed the enzyme.

(*d*) *Substrate Concentration*: The Michaelis constant (K_m) for the enzyme-nitrate complex, as estimated by the method of Lineweaver-Burk, was 7.5×10^{-5} *M*.

(*e*) *Electron Donors*: The rates of nitrate reduction were compared among redox-dyes in their saturated level of reduced forms (in the presence of a small amount of dithionite). Table VIII shows that reduced forms of MV, Ps, and nile blue were effective, whereas neutral red and those of the other dyes of higher potential were far less effective. MVH, the one-electron donor, was especially the most effective.

(*f*) *Heavy Metal Constituent of NaR, the Terminal Enzyme*: The fact that NaR activity of the purified preparation was neither inhibited by amytal, quinine, dicumarol, HOQNO at all nor activated by FAD, FMN, menadione or Fe^{++} proves that the purified preparation has much simplified system of electron tranfer to nitrate. Thus, without the participation of flavin, menadione, Fe^{++} and cyt. b_1, the electrons of PsH_2 or MVH seem to be transferred directly to NaR. The same seems to be true for the particulate NaR assayed with PsH_2.

On the other hand, as seen from Table IV, KCN and NaN_3 always produced the profound inhibition of the same extent regardless of the nature of the electron donors or preparations indicating the presence of a heavy metal component in NaR*: the terminal enzyme.

No significant difference in inhibition was observed with purified preparation between PsH_2 (the 2-electron donor) and MVH (the one-electron donor) (Table IV).

No inhibitory action of CO in the dark eliminates the possibility of participation of a CO-binding electron-transferring component in the whole system.

The enzyme could be inactivated by dialysis against 3×10^{-4} *M* KCN in 0.03 *M* phosphate buffer of pH 6.8 containing glutathione (3×10^{-4} *M*). Removal of cyanide by subsequent dialysis gave a preparation with a markedly lowered activity (35 per cent). However, the activity of the enzyme was not restored by the preincubation with salts of Fe^{++}, Fe^{+++}, MoO_4^{--}, Mn^{++}, Zn^{++} and Co^{++} singly or in various combinations at $10^{-3}\sim10^{-4}$ *M* final concentrations.

DISCUSSION

As previously reported from this laboratory, the particulate NaR system of *E. coli* (complex medium cells) was shown to be nitrate respiration type and the particulate form has been assumed to be characteristic for this type of nitrate reduction in respect of the mitochondria-like systems of aerobic respiration (*2*).

On the other hand, NaRs of nitrate assimilative organisms (*e.g. Neurospora* and soybean leaves (*11*)) were known to be in soluble forms*.

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* Recently, using the solubilized and highly purified preparation obtained from the particulate fraction of anareobically grown *E. coli* of the same strain, the terminal enzyme was shown to be a metalloflavoprotein (*10*).

The present soluble DPNH-NaR from the same strain of *E. coli* (synthetic medium cells) seems to be quite similar to the particulate DPNH-NaR system from the complex medium cells in respect of cyt. b_1 participation and non-assimilative nature though this is a soluble system. Thus, the soluble DPNH-NaR seems to be still nitrate respiration type.

The presence of the similar DPNH-NaR of the metallo enzyme nature, though far less active, was also confirmed in the $110{,}000 \times g$ supernatant from the complex medium cells**. The particulate DPNH-NaR from the complex medium cells has not been successfully solubilized (*2*), whereas the present DPNH-NaR could be easily extracted in an active state. In the present case, however, the DPNH-cyt. b_1 reductase activity could be easily eliminated but cyt. b_1 was still present after the 10-fold purification. On the contrary, Kinsky *et al.* (*13*) showed that in a highly purified preparation of *Neurospora* TPNH-NaR no cytochromes but a strong TPNH-cyt. c reductase activity was associated. The close association of some cytochrome with NaR seems to be also characteristic for NaR system of the nitrate respiration type and the similar fact have been reported in the case of cyt. b_1 preparation from Pseudomonad with NaR activity (*14*) and purified NaR from *Achromobacter* (*15*). DPNH-NaR activity was markedly inhibited by molecular oxygen, probably being due to the competition with active and cyt. b_1-containing DPNH oxidase activity present in a soluble form.

Menadione participation in the soluble DPNH-NaR may give a physiological significance to the menadione reductase system of *E. coli* which has not been physiologically uncharacterized (*16*). The present result was supported by the similar experiments on *E. coli* NaR by Wainwright (*17*) and Medina *et al.* (*18*). Medina *et al.* reported the restoration of atebrine inhibition on particulate DPNH-NaR of *E. coli* by the addition of not FAD but menadione and rejected the participation of FAD.

Fe^{++} participation in the soluble DPNH-NaR probably performing its valence change was demonstrated. The functional position of Fe^{++} in the electron transport system can be tentatively put between DPNH and cyt. b_1. Mahler *et al.* claimed the iron participation in the animal DPNH-cyt. c reductase (*19*). Weber *et al.* (*20*) reported the inorganic iron reduction by flavoproteins and non-enzymic reduction of cyt. c by Fe^{++} in the presence of citrate using mammalian and bacterial DPNH-cyt. c reductase systems. Iron participation between some flavoprotein and cytochrome seems to be general. In fact, the evidence for iron participation in NaR system of *E. coli* has been reported by Erkama (*21*) and Wainwright (*17*). Recently, Sadana *et al.* (*15*) also reported an iron participation between flavin and c type of cytochrome in the *Achromobacter* DPNH-NaR from nutritional and inhibition (photo-reversible CO inhibition) studies. In their experiment, however, the relationship between some CO-binding iron compound and c type cytochrome observed in their purified preparation remains obscure. It seems likely that Fe^{++} is functioning in the form of an iron-citrate or pyrophosphate complex presumably having redox-potentials or steric requirement different from Fe^{++} iron which are favorable to the enzymic reaction of soluble NaR with DPNH.

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* Recently, DPNH-NaR activity in nitrate-assimilating *Azotobacter vinelandii* (O strain) was demonstrated to be a particulate system (*12*).

** Unpublished data, Ohmachi *et al.*

The inhibition produced by HOQNO on the soluble DPNH-NaR seems to have its action site on the oxidation of cyt. b_1 rather than the reduction in respect of no restoration of the inhibition by menadione or D-α-tocopherol. This possibility is directly supported by Sato's confirmation* obtained by using NaR system (from succinate to nitrate) of *E. coli B* sonicate, HOQNO (about 10^{-5} M) and by means of sensitive double-beam spectrophotometer. The discrepancy from Lightbown's proposal (*22*) that HOQNO may inhibit the reduction site of cyt. b_1 in succinic oxidase system of *E. coli* may presumably be due to the difference of the strains and the growth conditions.

The fact that HOQNO inhibition remains at 70–80 per cent and never reaches 100 per cent even if the concentration is raised to 3×10^{-5} M may suggest that the possible functioning of a HOQNO-insensitive mechanism involving no cyt. b_1 in the soluble extract. The present situation is the same as already claimed by Sato (*23*) and Iida *et al.* (*2*). The main sequence of electron transfer for nitrate reduction in the synthetic medium cells, however, seems to consist of the soluble system (DPNH-NaR) and the particulate system (DPNH-NaR and formate *etc.* -NaR) both containing cyt. b_1 and equally active. On the basis of these considerations, it is concluded that the following proposed scheme (Fig. 6) of electron transport system is essential

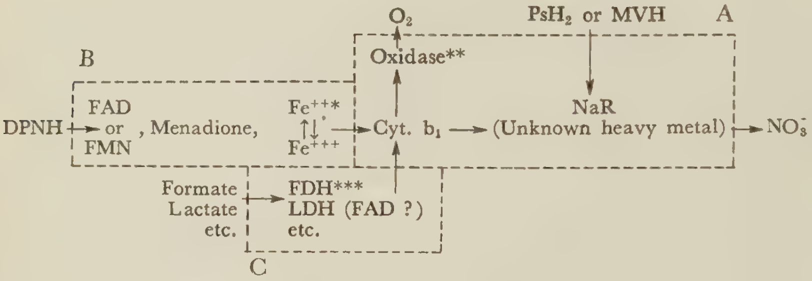

FIG. 6. The scheme of electron transport system to nitrate in *E. coli* Yamaguchi synthetic medium cells.

A: purified preparation, A+B: the soluble extract, A+B+C: particulate fraction.

* In the case of particulate DPNH-NaR, iron participation has not yet been confirmed.

** It has not been elucidated whether cyt. b_1 oxidase is present in the purified preparation or not.

*** Lactic dehydrogenase.

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* Personal communication from Dr. R. Sato.

for nitrate reduction of synthetic medium cells of *E. coli* Yamagutchi.

Yamagutchi strain failed to grow in completely synthetic media by assimilating nitrite presumably due to its less tolerance to nitrite toxity than that of nitrite-assimilating mutant of *E. coli* reported by McNall *et al.* (*24*). It has been demonstrated, by means of a sensitive recording spectrophotometer, that the living cells of *E. coli B* (which had preliminarily been confirmed to have cyt. b_1 containing NaR system when aerobically grown in a complex medium) have the distinct system without cyt. b_1 participation when aerobically grown by assimilating nitrate in the same synthetic medium (but no yeast powder)*. The above change in *E. coli B* (which is presumably more tolerant to nitrite toxity than the Yamagutchi strain) from nitrate respiration type to nitrate assimilation type could be understood to be due to the change of growth conditions. In the present case of *E. coli* Yamagutchi, under the similar change of growth conditions, the distinct mode of change of NaR system—from particulate to more soluble system of still nitrate respiration type—was demonstrated.

SUMMARY

1. *E. coli* (Yamagutchi strain) aerobically grown in the synthetic medium never assimilated nitrate. About 30 per cent of the NaR activity (assayed by using PsH_2) of the whole cells was found in the soluble extract (107,000×g supernatant).

2. The soluble extract contained DPNH-NaR of nitrate respiration type which consisted of flavin, menadione, Fe^{++}, cyt. b_1 as intermediary electron carriers (Fig. 6) and of sulfhydryl group. The cyanide and quinoline-*N*-oxide-sensitive DPNH oxidase probably involving cyt. b_1 was also found in the soluble extract.

3. In the soluble extract, Fe^{++} could enzymically donate its electron to nitrate probably via cyt. b_1 with the comparative rate as from DPNH.

4. Formate-NaR was never demonstrated in the soluble extract but resided only in the particulate fraction. This formate-NaR and DPNH-NaR both residing in the particulate fraction were cyt. b_1-containing and of the same character as previously reported one existing in the complex medium cell (*2*).

5. The soluble NaR preparation purified 10-fold from the crude extract could reduce nitrate by only using some reduced dyes. This purified preparation still contained a small amount of cyt. b_1 and cyt. b_1-NaR activity but no DPNH-cyt. b_1 reductase activity.

6. The purified enzyme (assayed by using PsH_2) had K_m of $7.5\times10^{-5}\,M$ for enzyme-nitrate complex, pH optimum at 6.4 and was activated by citrate or pyrophosphate. This terminal enzyme had some CO-insensitive heavy metal constituent.

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* These experiments were carried out by one of us (S.T.) during his stay in Dr. B. Chance's laboratory.

He wished to express his gratitude to the provided facility.

The authors wish to express their sincere thanks to Prof. F. Egami for his valuable criticism and also to Messrs. K. Iida and K. Ohmachi for their discussions and generous assistances in carrying out the experiments.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

THE STRUCTURE OF CYTOCHROME C

VII. CHROMATOGRAPHIC SEPARATION OF PEPTIDES OBTAINED BY TRYPTIC HYDROLYSIS OF CYTOCHROMES C FROM HORSE-HEART AND BAKER'S YEAST

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(Received for publication, March 12, 1959)

In the previous paper (*1*), the authors reported the amino acid composition of cytochromes c from various sources, which had been determined by the method of Moore and Stein. Therein was demonstrated the close resemblance in amino acid composition between mammalian and yeast cytochromes c. Similarity in some physicochemical properties between horse-heart and yeast cytochromes c. was previously investigated by one of the authors (S. M.) (*2*), and in so far as tested, both cytochromes showed the same activity.

The present paper deals with some results of the chromatographic separation of peptides obtained by tryptic hydrolysis of cytochromes c from horse-heart and baker's yeast. This work was carried out to obtain further informations on the structure of cytochromes c with the interest from the view point of comparative biochemistry and as a preliminary step towards the elucidation of the peptide structure.

EXPERIMENTAL

Horse-heart cytochrome c was purified as described in the previous paper (*3*). Yeast cytochrome c was purified according to the procedure of Hagihara *et al.* (*4*). 20 to 200 mg. of each cytochrome c was digested with one-hundredth the amount of crystallized trypsin in 0.2 M sodium phosphate buffer of pH 7.0 at 25° for 25 hours at a substrate concentration of 0.5 per cent. In preliminary experiments the progress of the digestion was followed by the ninhydrin method of Moore and Stein (*5*) and the completeness of the digestion was checked by adding small amounts of trypsin to the reaction mixture and keeping it to stand for a few more hours, when no appreciable increase in the ninhydrin color was observed. The reaction was stopped with 2 N HCl by bringing the pH of the reaction mixture to 2.2. A typical digestion curve for horse-heart cytochrome c is given in Fig. 1.

The chromatographic separation of peptides thus obtained was carried out on a column of Dowex-50×2(200–400 mesh, Na form) (150×1.0 cm.) according to the procedure of Moore and Stein (*5*). Hydrolysate from 20 to 40 mg. of each cytochrome c was loaded on the column. The effluents were collected in 2 ml. fractions and analysed by the ninhydrin method. Parts of the effluents were also subjected to the ninhydrin analysis

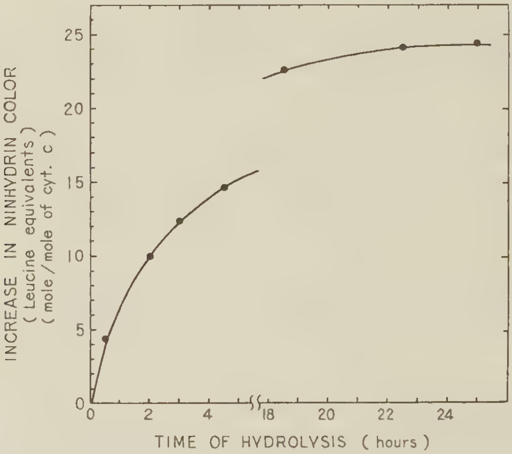

FIG. 1. Tryptic hydrolysis of horse-heart cytochrome c. Cytochrome c 0.5 %, trypsin 0.005 % pH 7.0 (0.2 *M* sodium phosphate buffer), 25°.

after hydrolysis with 2.5 *N* NaOH at 90° for 3 hours. The chromatograms are given in Fig. 2. The separations were repeated several times and reproducible results were obtained, including preparative-scale separation of peptides from horse-heart cytochrome c (about 200 mg.) on a column of Dowex-50×2(150×2.0 cm.).

RESULTS AND DISCUSSION

As shown in the chromatograms (Fig. 2). peptides were successfully separated into 21 peaks with horse-heart cytochrome c and 17 peaks with yeast cytochrome c. With yeast cytochrome c, one peak was negative to the ninhydrin reaction and detected after alkaline hydrolysis. In the previous study (*1*), it was shown that horse-heart cytochrome c contained 18 moles of lysine and 2 to 3 moles of arginine residues per mole and yeast cytochrome c, 15 moles of lysine and 3 moles of arginine residues per mole. Considering the substrate specificity of trypsin, these results would suggest that the digestion proceeded rather strictly according to the enzymes specificity and that peptides produced were separated quite well on the column. The two chromatograms are considerably different from each other. Great differences were also observed in paper-chromatograms of the peptides from the two cytochromes c. These results might suggest that there would exist rather remarkable differences in the peptide structure between horse-heart and yeast cytochromes c, though they are in close resemblance in the amino acid composition, as well as in some physico-chemical properties and the catalytic function.

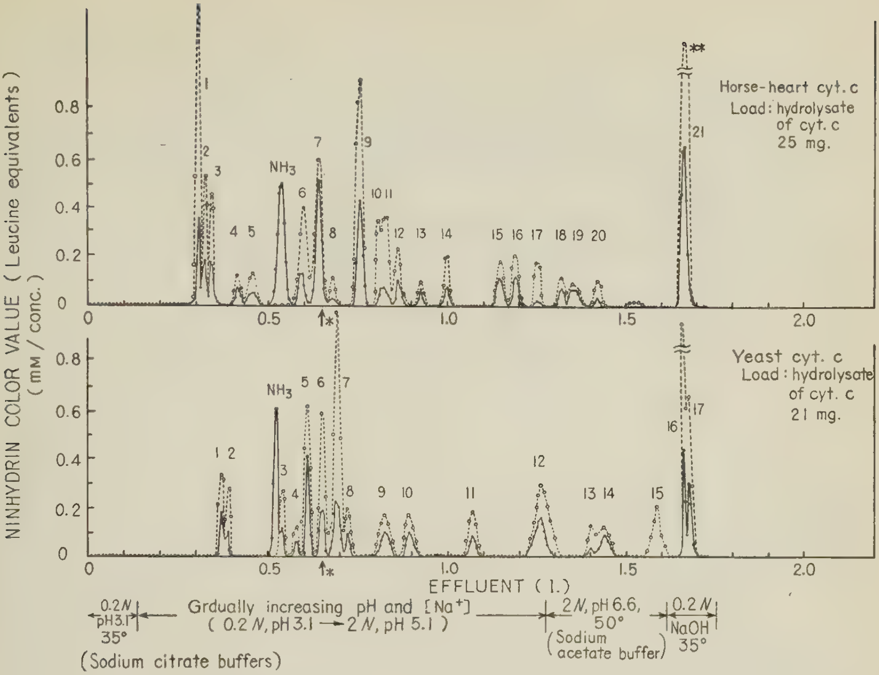

FIG. 2. Separation of peptides on a column of Dowex-50×2(150×1.0 cm.).

Resin; Dowex-50×2(200–400 mesh), Na-form, Column; 150×1.0 cm., Mixing chamber; 600 ml. in volume, Initial flow rate; 2 ml./fraction, Analysis; the ninhydrin method: Sample 0.5 ml. from each fraction, optical densities at 570 mμ were read before (the solid line) and after (the dotted line) alkaline hydrolysis with 2.5 *N* NaOH solution at 90°, for 3 hours.

* . . . 1 *N* sodium citrate buffer of pH 5.1 in the upper chamber was replaced with 2 *N* buffer of pH 5.1 at 0.65 effluent liter.

** . . . In the peaks—No. 21(horse), No. 16 and 17(yeast) were involved haem-containing peptides and the effluents were colored red-brown.

SUMMARY

1. Cytochromes c from horse-heart and baker's yeast were hydrolysed with trypsin, and the resulting peptides were successfully separated on a column of Dowex-50×2(150×1.0 cm.) into 21 peaks for the former and into 17 peaks for the latter with good reproducibility.

2. Chromatograms of the peptides from both cytochromes c were quite different from each other, which was suggestive of some great differences in the peptide structure.

The authors acknowledge their thanks to Prof. S. Akabori and Prof. H. Yoshikawa for constant guidances during the cource of study. Thanks are also due to Prof. F. Egami for valuable discussions.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

STUDIES ON ENZYMATICALLY ACTIVE FRAGMENTS OF PEPSIN

I. PREPARATION AND NATURE OF FRAGMENTS WITH PEPTIC ACTIVITY

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Although pepsin was crystallized by Northrop (*1*), it was certainly not regarded as an enzymatically homogeneous protein (*2–11*). It was found that crystalline pepsin contained gastric cathepsin and other proteolytically inactive substances. This seems to suggest that enzymatically pure pepsin might be a protein with a molecular weight of less than 35,000.

Commercial pepsin preparations are mixtures of gastric enzymes with pepsin as a main component and also cathepsin and gelatinase (*12*, *13*). Beside these three proteinases, *Brücke* pepsin (*14*), nonprotein pepsin, has been shown to be present in commercial pepsin preparations (*15*). *Brücke* pepsin was assumed to be a different proteolytic enzyme from pepsin (*15*, *16*). Such an enzyme of non-protein nature, if it really exists, must be an extremely useful substance for the study of the relationship between the structure and function of enzyme proteins. To study the structure of the active center of an enzyme, it is desirable to prepare an enzyme protein having the minimum molecular weight essential for its activity.

To this end an attempt was made to isolate *Brücke* pepsin from a commercial pepsin preparation by the method of Kraut and Tria (*15*). Purification was however unsuccessful probably due to a low content of *Brücke* pepsin in the pepsin preparation.

It is of interest that Perlmann (*17*) has isolated at least two active dialysable fragments from an autolysate of crystalline pepsin at pH 5.6. This suggests that *Brücke* pepsin might be a product of spontaneous degradation of pepsin during the preparation of the latter. Therefore, an attempt was made to prepare this active degradation product from an autolysate of pepsin.

MATERIALS AND METHODS

Pepsin: A commercial pepsin preparation from N.B.C., 1 : 10,000 U.S.P. was employed.

Determination of Proteolytic Activity (*18*)—(*a*) *Estimation of Peptic Activity*: A 0.6 per cent *Hammersten*'s casein solution of pH 1.8 was used as substrate. The procedure for the estimation was as follows: 1 ml. of enzyme solution previously warmed at 35° for 5 minutes was added to 5 ml. of casein solution at 35°. After a definite period of incubation at 35°, 5 ml. of 0.44 *M* TCA solution was added to stop reaction. After keeping the reaction mixture for 30 minutes at 35°, it was filtered through hardened filter paper, Tōyōroshi No. 4. Then 5 ml. of 0.4 *M* Na_2CO_3 solution and 1 ml. of 1/5 diluted Folin reagent (*19*) were added to 2 ml. of the filtrate and the solution was kept at 30° for 30 minutes. The intensity of the colour developed was measured with a Beckman spectrophotometer at 660 mμ. In this paper the protease activity is expressed as mg. of tyrosine produced per mg. of nitrogen of the enzyme.

(*b*) *Estimation of Stomach Catheptic Activity*: Buchs (*20*) has shown that after heating at 70° for 30° minutes, cathepsin was still active, while pepsin was completely inactivated. Thus, it is possible to estimate activity separately from peptic activity after heating the enzyme solution at 70° for 30 minutes. The subsequent procedure for the estimation of the catheptic activity was the same as that described above for peptic activity except that the incubation was at 65° instead of 35°.

Estimation of Enzyme Protein Concentration—The modified Folin method of Lowry (*21*) was used.

Continuous Paper Electrophoresis—A modification of the apparatus of Grassmann and Hannig's (*22*) was used. To increase the cooling efficiency, the buffer solution used for cooling the electrodes was supplied directly and rapidly from outside the case enclosing the apparatus. Tōyōroshi filter paper No. 2 and No. 50 were employed. To cover the desired pH range, HCl-NaCl (pH 1.8), phthalate (pH 2.2-4), and McIlvaine's acetate (pH 4-6) buffers were employed. The ionic strength of the buffers was between 0.07 and 0.1.

Autolysis—To avoid possible destruction of the active fragments, the autolysis was performed in a collodion membrane immersed in a buffer solution. Thus the permeable autolysate moved gradually into the outer solution and was obtained as a dialysate. This "dialysis autolysis" appears to be valuable for obtaining an active fragment. Fig. 1

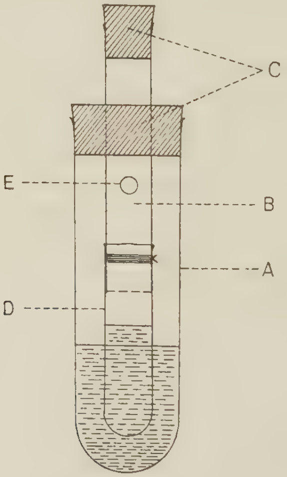

FIG. 1. Apparatus for dialysis autolysis.
A: large test tube, B: glass tube, C: rubber stoppers, D: collodion membrane, E: a small hole.

shows the dialysis autolysis apparatus used in the present study. A is a large test tube of about 3 cm. diameter, B is a glass tube to support the collodion membrane, D. Both glass tubes were closed by rubber stoppers, C. E is a small hole to maintain the same air pressure inside and outside the membrane. Two volumes of outer solution were used per volume of inner enzyme solution. Autolysis of a 0.6 per cent crude pepsin solution was carried out at a definite temperature for a definite period. Small amounts of toluene were added to both inner and outer solutions to prevent bacterial growth.

Fractionation of Active Fragments with Alcohol—Two types of dialysates obtained by 94 hours' autolysis in buffer solutions of ionic strength 0.1 and 0.01 were employed.

The pH of the solution was adjusted to 1.80 and a measured volume of cooled 95 per cent alcohol was added to 10 ml. of the solution to obtain the desired concentration of alcohol. When the solution was kept at 5° for 24 hours, a precipitate appeared was centrifuged and washed with aqueous alcohol of the same concentration. Then determinations were made of the nitrogen content and enzymatic activity of the precipitate after dissolving it in 10 ml. of HCl-NaCl buffer solution of pH 1.8. To the supernatant a definite volume of 95 per cent alcohol was again added. The precipitate formed was separated in the same way as mentioned above. The nitrogen content and enzymatic activity of each precipitate were measured.

Fractionation of Active Fragments with Trichloracetic Acid (*TCA*)—To 2 ml. of the dialysate was added a known volume of previously warmed 50 per cent TCA solution and the total volume was made up to 10 ml. with water. The pH was adjusted to 1.8. After standing at 35° for 30 minutes, the precipitate formed was removed by filtration. The nitrogen content and enzymatic activity of the filtrate was determined after the TCA was removed from the filtrate by washing with four 10 ml. aliquots of ether.

RESULTS AND DISCUSSION

Proteinase Activities in the Crude Pepsin Preparation—To examine what type of proteinase activities were present in the crude pepsin preparation, fractionation was performed by the continuous paper electrophoresis. The development of electrophoretic pattern on a 21×23 cm. sheet of filter paper (Tōyōroshi No. 50) was carried out by using 0.07 μ phthalate buffer of pH 3.60 and applying a 4.5–4.9 mA. current at 120 volts.

The electrophoretic pattern appearing after treatment with ninhydrin reagent is shown in Fig. 2-a.

A similar pattern was also obtained at pH's 2.16 and 4.80 under different electric conditions. However, the position of each component was not exactly the same at each pH. For instance, a component, which flowed into fraction 5 at pH 3.60, shifted to fraction 8 at pH 4.80. Fig. 2-b shows the nitrogen content and the peptic and catheptic activities of each fraction obtained at pH 3.6. The proteinase activity of fractions obtained at pH 2.16 was remarkably low. This suggests that peptic autodigestion was taking place during electrophoresis.

By the heat test described under methods fraction 22, which moved rapidly to the anode, was found to have peptic activity. Fractions 17 to 20 were found to have a mixture of peptic and catheptic activities, since heating at 70° did not completely destroy activity.

A large part of the pepsin preparation was isolated in fractions 13 to 15,

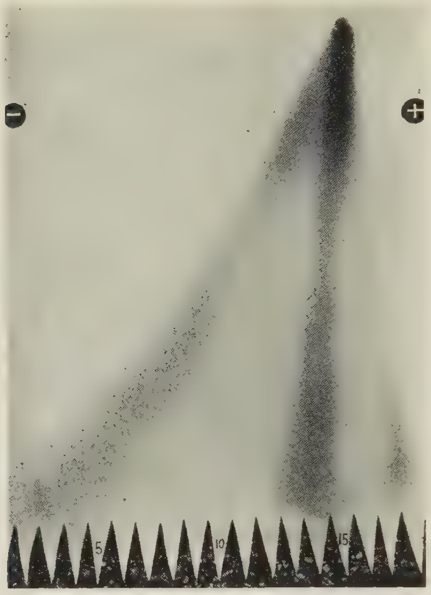

FIG. 2-a. A continuous paper electrophoretic pattern of crude pepsin. Conditions: HCl-potassium-biphthalate buffer, pH 3.6, 0.07 μ, 120 v., 4.5-4.9 mA.; Tōyōroshi No. 50, 21 × 23 cm.

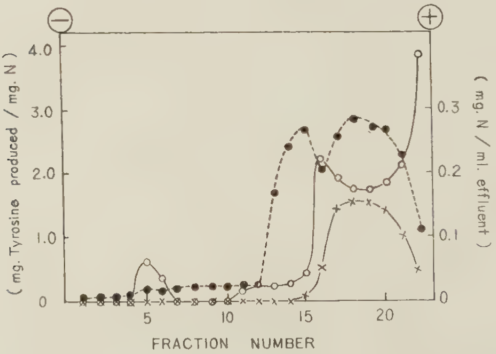

FIG. 2-b. Fraction diagram of crude pepsin by continuous paper electrophoresis. ----●----, Nitrogen contents; —○—, Peptic activity; —×—, Catheptic activity. Conditions: See Fig. 2-a.

so that the nitrogen content of these fractions was the highest. However, the proteolytic activity was so low that these fractions must be considered to contain enzymatically inactive proteins.

A small amount of material was separated in fractions 5 and 6. The combined fractions were found to have an ability of digesting casein at pH 1.8, and were inactivated by heating 70°. Therefore, this substance seems to have peptic activity. However, it appears to be a more positively charged substance than pepsin at this pH.

The three components contained in fractions 22, 17–20 and 13–15, respectively, seemed to correspond to the three components which have been obtained from crystalline pepsin by Merten *et al.* (*13*). However, the fourth component contained in fractions 5 and 6 was not reported by them. This component therefore might exist only in a crude pepsin preparation and not in crystalline pepsin. At present, there is no experimental evidence to infer that the component is identical with *Brücke* pepsin.

These results, however, suggest the possibility of digesting pepsin with the accompanying cathepsin during the autolysis of a crude pepsin preparation. In other words, in the autolysis either of crystalline or crude pepsin, the mutual digestion of these two proteinases has to be considered apart from the autolysis of the homogeneous enzyme.

Fractionation of Peptic-Active Fragments in Dialysis Autolysis of a Crude Pepsin Preparation—A study was made of the effects of buffers, time, and temperature on the formation of enzymatically active fragments of pepsin during autolysis.

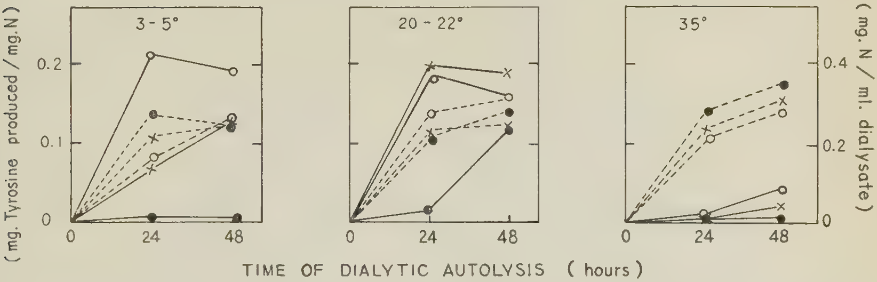

FIG. 3. Nitrogen contents and protease activity in dialysate obtained by dialysis autolysis of crude pepsin in various buffer solutions of ionic strength of 0.1 at different pH's and various temperatures. (●) pH 2.00 (HCl-NaCl buffer), (○) pH 4.05 (acetate buffer), (×) pH 5.90 (phosphate buffer). Dotted and solid line represents nitrogen contents and activities respectively.

Experiments were first performed in various buffers of different pH's and the same ionic strengths at different temperatures (3–5°, 20–22°, and 35°), for various periods of time. The nitrogen contents and peptic activity of the dialysates were determined after various periods of autolysis. The results are shown in Fig. 3.

The dialysate obtained by autolysis at pH 2 had low peptic activity at all temperatures and in all buffers, despite the fact that the quantity of nitrogen was equivalent to that obtained by autolysis at higher pH's. This suggested that at pH 2, autodigestion of pepsin occurred so rapidly that it was almost impossible to control the degree of autolysis. Therefore, autolysis at such a pH appears to be unsuitable for obtaining active fragments of pepsin.

At pH's higher than 4, the peptic activity formed in the dialysate was also low. At these pH's, the self-inactivation of pepsin and/or a rapid degradation of pepsin by activated cathepsin might account for the low yield of active pepsin fragments.

The effect of the ionic strength of the buffers on the formation of active substances in the dialysate was studied at 3–5° and at pH 4.0 comparing 0.1 μ with 0.01 μ acetate buffer. As shown in Fig. 4, very little activity was found in the dialysate obtained from a 0.01 μ buffer solution. Since the quantity of nitrogen in the dialysate obtained from 0.01 μ buffer solution was only slightly smaller than that from a 0.1 μ buffer solution, it seems that active fragments are hardly produced by autolysis under the above conditions.

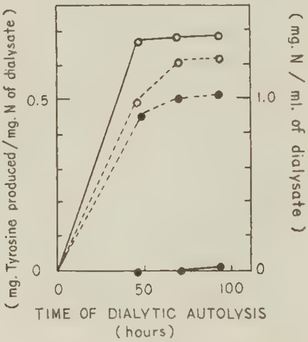

FIG. 4. Dialysis autolysis of crude pepsin at 3–5° in acetate buffer solutions of pH 4.05 at different ionic strengths, 0.1 (○) and 0.01 (●). Dotted and solid lines represent nitrogen contents and activities respectively.

It was also found that the activity of the dialysate increased with time and reached a maximum value within 48 hours in all experiments.

Therefore, it can be concluded that the active fragment of pepsin was produced most efficiently by dialysis autolysis of crude pepsin in 0.1 μ McIlvaine's acetate buffer of pH 4 at 3–5° for 48 hours.

Nature of the Permeable, Active Fragments of Pepsin in the Dialysate—To elucidate the chemical entity of permeable peptic activity in the dialysate, fractionation of the dialysate with alcohol and TCA has been performed. In addition, the pH dependance of the activity and the heat stability at various pH's were studied.

(*a*) *Fractionation of the Active Substances in the Dialysate with Alcohol*: Fig. 5 shows the peptic activity during alcohol fractionation at pH 1.8 of a dialysate and the crude pepsin solution.

Only one active material was obtained from a crude pepsin solution, which was precipitated in 20 per cent alcohol. However, in the dialysate obtained with 0.1 μ buffer of pH 4 two active peaks were observed at the higher alcohol concentrations, *i.e.* 30–40 and 80 per cent.

It is known that crystalline pepsin is soluble in both 20 and 70 per cent aqueous alcohol at pH 4, but insoluble in 20 per cent alcohol at pH 1.8–2

(*23*). Therefore, so far as the precipitation is concerned, the active substance precipitated from the crude pepsin solution seems to be identical with crystalline pepsin. However, the active substances from the dialysate were precipitated at different concentrations. The one fraction, precipitated at 30–40 per cent alcohol concentration, seems to be slightly more soluble in aqueous alcohol than the native pepsin. The other fraction which was precipitated at a higher alcohol concentration is much more soluble. Therefore, both active substances in the dialysate are assumed to differ from the native pepsin.

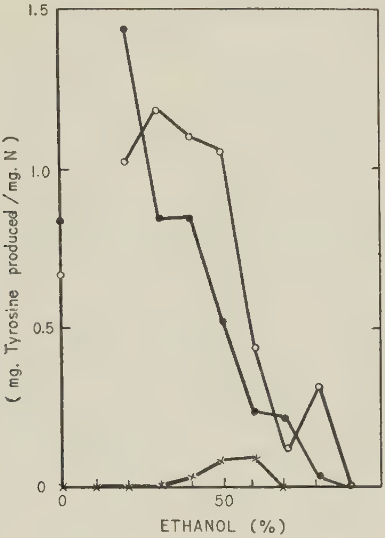

FIG. 5. Fractionation of a crude pepsin solution and its autolytic dialysate with ethanol.

Activity in precipitatates resulted from 30-minute standing at 5° following addition of ethanol to dialysate ($\mu=0.1$) (—○—) and to crude pepsin solution (●). Activity in an identical precipitate from dialysate ($\mu=0.01$) which was left standing for 24 hours at 5° (—×—).

On studying the active substances in the supernatants of the alcoholic fractionation, evidence was also obtained which indicated that the active substance precipitated from 80 per cent alcohol was not identical with native pepsin. As is shown in Fig. 6, very high activity was observed in the 10 per cent alcohol soluble part of the dialysate, while no activity was found in the corresponding fraction of crude pepsin. These facts suggest that there are at least two types of substances having peptic activity in the autolysis dialysate in 0.1 μ acetate buffer possibly with different molecular weights. It is likely that these were fragments produced from the enzymes, since they were not initially present in the crude pepsin preparation. However, these experiments did not show whether these fragments originated from pepsin or cathepsin. As was mentioned above, the ionic strength of the buffer solution affected the amounts and probably the type of the active substance produced in the dialysate. For instance, as is shown in Fig. 5, no active substance precipitating at 30–40 per cent alcohol concentration is produced in a buffer of 0.01 ionic strength, in contrast to that of 0.1 ionic strength. In 0.01 μ acetate buffer solution of pH 4 a precipitate having considerable activity appeared at 50–60 per cent alcohol concentration. Therefore, in the preparation of an active fragment of pepsin, the ionic strength as well as the pH of a solution should be taken into consideration.

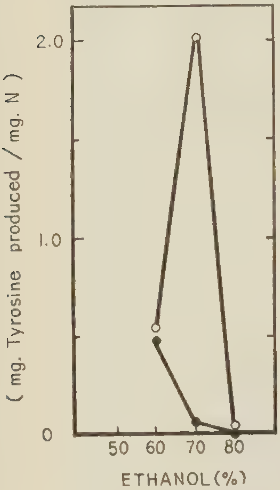

FIG. 6

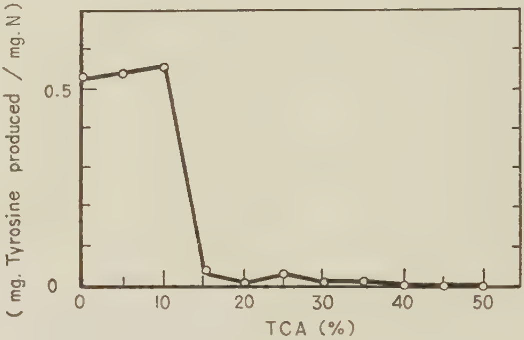

FIG. 7

FIG. 6. Protease activity remained in supernatant on addition of ethanol to dialysate (μ=0.1) (—○—) and to crude pepsin solution (—●—). Activity was measured after standing for 30 minutes at 5°.

FIG. 7. Protease activity remained in supernatant obtained by treating dialysate with TCA.

(*b*) *Fractionation of the Active Fragments with TCA*: The peptic activities of supernatants obtained by progressively increasing the concentration of TCA are given in Fig. 7. In the supernatant from 10 per cent TCA the activity per mg. of nitrogen was greater. This appears to be due to the precipitation of inactive proteins by TCA. At a 15 per cent TCA concentration a large part of the activity was precipitated. However, there was a small amount of activity which was still soluble in a 25 per cent TCA solution.

Thus, it is also probable that there are two types of active substances in the dialysate obtained by using 0.1 μ buffer of pH 4.

(*c*) *The pH-Activity Curve of the Two Types of Active Substances*: The active substances obtained either from a 30 per cent or 80 per cent alcohol solution were examined for the pH dependence of their activation. As is shown in Fig. 8, both active substances showed the highest activity at approximately pH 1.5. This suggests that these active substances are fragments originating chiefly from pepsin. However, an active fragment derived from cathepsin may be present with these pepsin fragments, since a considerable activity was also observed at pH 3.5. It seems to be hard to obtain an active fragment from pepsin alone by this method.

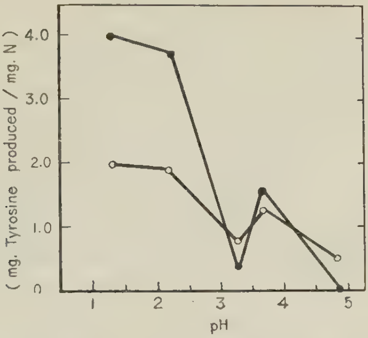

FIG. 8. Effect of pH on the activity of the components precipitated by 30 per cent (—●—) and 80 per cent (—○—) ethanol.

The pH optimum of the substance obtained with 80 per cent alcohol was much wider than that obtained from 30 per cent alcohol solution. Since the substance obtained from 80 per cent alcohol is probably degraded to a smaller enzyme molecule, the degradation of the enzyme molecule seems to results

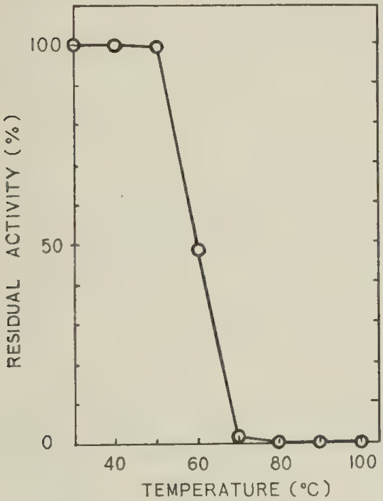

FIG. 9

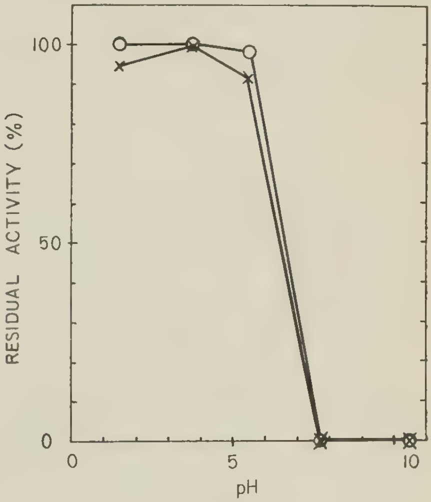

FIG. 10

FIG. 9. Heat stability of dialysable active fragments at pH 4.05.

FIG. 10. pH-stability of dialysable active fragments at 20°. Activity was measured after leaving at each pH for 48 hours (—○—) and 200 hours (—×—).

first in a decrease in the specificity of the enzyme, *i.e.* pH dependency and substrate specificity, and subsequently in a loss of activity.

(*d*) *Heat Inactivation of the Active Fragments*: Fig. 9 shows the effect of heat on the activity of the fragments. Upon heating for 30 minutes, no inactivation occurred until 50°. At 60° half of the activity was lost. At temperatures higher than 70° there was almost complete inactivation.

(*e*) *The Stability of the Active Fragments at Various pH's*: The dialysates were kept at different pH's either for 48 or for 200 hours at 20°. After adjusting the pH to 1.8 the peptic activity was determined with casein as substrate. Fig. 10 indicates that this activity was stable below pH 5.5, and even to pH 1.5, where crystalline pepsin was not stable.

Although it was not possible to isolate *Brücke* pepsin from a crude pepsin preparation, it was found that permeable fragments having peptic activity were obtained by a limited autolysis of a crude pepsin preparation. This seems to suggest that *Brücke* pepsin is possibly a degradation product of pepsin produced during the preparation of the latter. By controlling the conditions for the preparation, several types of active fragments were obtained. One of them had a remarkable solubility in a aqueous alcohol and TCA solution. It is of interest that the activity of this remarkably soluble fragment was not affected by pH. In this connection an active fragment having a pH optimum at 3.5 is certainly not regarded as a fragment produced from cathepsin. In active fragments of highly degraded enzyme, the optimum pH for activity does not always to be identical with the original optimum pH of the intact enzyme. It may be possible to assume that an active fragment of pepsin has its optimum pH at 3.5.

SUMMARY

1. A commercial pepsin preparation contained pepsin, cathepsin, and another unknown peptic activity.

2. Several types of fragments with peptic activity were obtained by dialysis autolysis of the crude pepsin preparation. The type of active fragment varied with the conditions of autolysis, particularly, with the ionic strength and pH of the solution.

3. These fragments probably have smaller molecular weights than the native pepsin, although their molecular weights were not determined in the present study. The properties of these fragments were considerably different from those of the native pepsin. Degradation of pepsin appears to broaden the pH range for optimum activity.

4. No evidence for the identity of these fragments with *Brücke* pepsin is given.

We wish to express our sincere thanks to Prof. Y. Oshima, Kyushu University, for his helpful advice and kind support. It is a pleasure to acknowledge with thanks the valuable advice and helpful assistance of Prof. T. Tomiyama, Kyushu University, Dr. M. Ottesen, Carlsberg Laboratorium, and Dr. M. Sonenberg, Sloan Kettering

Memorial Institute for Research in Cancer and Allied Diseases, during preparation of the manuscript.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

STUDIES ON HOMOGENTISICASE*

II. STOICHIOMETRY OF THE REACTION AND NATURE OF REACTIVE GROUPS

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(Received for publication, March 30, 1959)

In a preceding paper (*1*), the role of ferrous ion which is the essential cofactor of homogentisicase has been investigated in some detail, using purified enzyme preparations. It has been also reported that the ferrous ion bound to the enzyme protein exchanges with exogenous Fe^{59} during the enzymatic catalysis. Crandall (*2*), Schepartz (*3*), and Knox and Edwards (*4*) have, on the other hand, demonstrated that the activation of the enzyme with ferrous ion can be enhanced by ascorbic acid or glutathione, and that the enzyme activity is strongly inhibited by SH-reagents such as mercuric chloride, *p*-chloromercuribenzoate and iodosobenzoate. Knox and Edwards (*5*) have further shown that the immediate product formed in the homogentisicase reaction is maleylacetoacetic acid. Using O^{18} as a tracer, Mason (*6*) has recently revealed that molecular oxygen is directly utilized in the oxidation of homogentisic acid.

The purpose of the present investigation is to obtain further information concerning the nature of the reactive groups of homogentisicase by studying the effects of reducing agents, SH reagents and tyrosinase on the enzymatic activity. A stoichiometric relationship among the components involved in this reaction has also been investigated.

METHODS

Homogentisicase was purified from acetone-dried powder of beef liver as described in the previous peper (*1*). Two kinds of preparations, namely, Fe^{++}-free and Fe^{++}-containing enzymes, were used throughout the experiments.

Tyrosinase was extracted from potato and partially purified by the method of Kubowitz (*7*). No protease activity in the preparation was found in the pH range tested.

Protein was determined by the use of Folin-Ciocalteu's phenol reagent (*8*).

Homogentisic acid (m.p. 146°) was isolated from alcaptonic urine as described previously (*1, 9*).

The amount of homogentisic acid was measured from the value of optical density at

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* This study was supported by a grant for scientific research from the Ministry of Education. A part of this work was reported at the 14th International Congress of Biochemistry held in Vienna, September 1958.

660 mμ according to the method of Brigg, as modified by Neuberger (*10*).

Maleylacetoacetic acid was determined at 330 mμ in a Beckman model DU spectrophotometer. Its absorption spectra were measured by a Carry model 14 recording spectrophotometer.

The assay of homogentisicase was made by the manometric method using a reaction mixture described previously (*1*).

RESULTS

Stoichiometry of Reaction—The fact that the product of homogentisic acid oxidation is maleylacetic acid was confirmed by measuring its absorption spectra. Fig. 1 is a reproduction of the absorption spectra measured at pH 1, 7 and 13 of the reaction product obtained in the present study. These spectra are identical with those described by Knox and Edwards (*5*) for maleylacetoacetic acid.

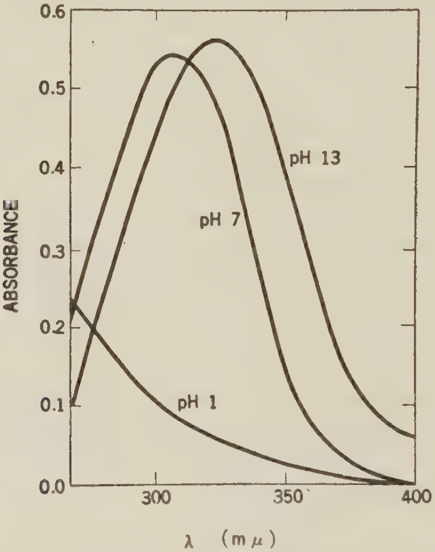

FIG. 1. Absorption curves of maleylacetoacetic acid (5×10^{-5} *M*) at pH 1, 7 and 13. The absorption maximum was observed at about 325 mμ at pH 13.

The stoichiometry of the homogentisicase reaction was examined in the following manner. After the oxygen uptake was determined manometrically at the time indicated, the reaction was stopped immediately by the addition of trichloracetic acid and that time was recorded. The content of the vessel was centrifuged, and the supernatant was subjected to the assay of substrate and product. As shown in Fig. 2, it confirms the results reported by Knox and Edwards (*11*), that for each mole of oxygen consumed, one mole of homogentisic acid disappeared and, at the same time, one mole of maleylacetoacetate was formed as expected from the following equation:

$$\text{Homogentisic acid} + O_2 \longrightarrow \text{maleylacetoacetic acid}$$

Furthermore, such a stoichiometric relation was continuously observed throughout the entire course of the reaction. It may, therefore, be concluded that the reaction proceeds either in one step in which the two oxygen atoms react with the substrate simultaneously, or in two steps in which the intermediate product is sufficiently labile to be rapidly converted to maleylacetoacetic acid.

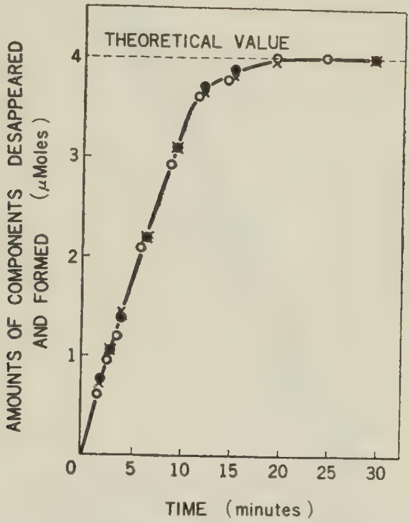

FIG. 2. Stoichiometric relationships in homogentisicase reaction. Oxygen uptake was measured manometrically at 30°, pH 6.8. Each vessel contained the Fe^{++}-free enzyme solution (3.4 mg. protein), $5\times10^{-4}\,M$ $FeSO_4$, $2\times10^{-3}\,M$ homogentisic acid, $4\times10^{-2}\,M$ phosphate buffer and distilled water to make the final volume 2 ml. At the time indicated, oxygen consumption was recorded and then the reaction was stopped by the addition of 0.1 ml. of 100 per cent trichloracetic acid to the reaction mixture. The content of the reaction vessel was contrifuged at 900×*g*. for 5 minutes, and the supernatant was diluted and used for the analysis of the amount of homogentisic acid remained or maleylacetoacetic acid formed.

○ : Value of moles of molecular oxygen disappeard.
● : Value of moles of homogentisic acid disappeared.
× : Value of moles of maleylacetoacetic acid formed.

Effect of Reducing Agents—Homogentisicase is known to be activated by reducing agents such as glutathione and ascorbic acid (*2*, *4*). Activation effected by these two reagents was confirmed in the present study.

As indicated in Fig. 3, the Fe^{++}-free enzyme preincubated with ferrous ion was markedly activated by the addition of glutathione and ascorbate as in the case of Fe^{++}-enzyme. In the absence of ferrous ion, however, the addition of the reducing agent alone showed no effect on the Fe^{++}-free enzyme reaction.

Fig. 4 shows the relationships between the degree of activation caused by various agents and their concentrations. Whereas Crandall (*2*) and Knox and Edwards (*4*) reported inhibition by cysteine on the enzyme

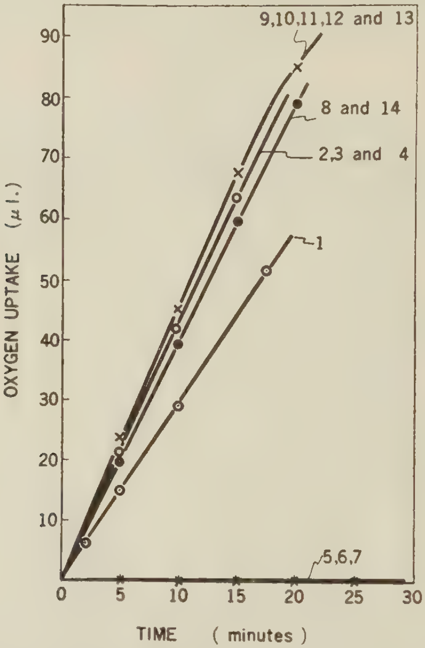

FIG. 3. Effect of glutathione and ascorbic acid at their optimal concentrations.

Assay was made manometrically at 30°, pH 6.6. Gas phase was air. Each vessel contained 0.5 mg. of the enzyme preparation, $2.0\times 10^{-3}\,M$ homogentisic acid, $4\times 10^{-2}\,M$ phosphate buffer, distilled water and the components described below. The total volume was 2 ml. Fe^{++}-free enzyme preparation :

1. $FeSO_4$, $1\times 10^{-3}\,M$
2. $FeSO_4$, $1\times 10^{-3}\,M$+ascorbic acid, $1.3\times 10^{-3}\,M$
3. $FeSO_4$, $1\times 10^{-3}\,M$+glutathione $1.3\times 10^{-3}\,M$
4. $FeSO_4$, $1\times 10^{-3}\,M$+ascorbic acid, $1.3\times 10^{-3}M$+ glutathione, $1\times 10^{-3}\,M$
5. Ascorbic acid, $1.3\times 10^{-3}\,M$
6. Glutathione, $1.3\times 10^{3}\,M$
7. No addition

Fe^{++}-containing enzyme preparation :

8. $FeSO_4$, $1\times 10^{-3}\,M$
9. $FeSO_4$, $1\times 10^{-4}\,M$+ascorbic acid, $1.3\times 10^{-3}\,M$
10. $FeSO_4$, $1\times 10^{-3}\,M$+glutathione, $1.3\times 10^{-3}\,M$
11. $FeSO_4$, $1\times 10^{-3}\,M$+glutathione, $1.3\times 10^{-3}\,M$+ ascorbic acid, $1\times 10^{-3}\,M$
12. Glutathione, $1.3\times 10^{-3}\,M$
13. Ascorbic acid, $1.3\times 10^{-3}\,M$
14. No addition

action, the amino acid was found to accelerate the reaction when it was supplied at suitable concentrations ($3\times 10^{-4}\sim 1\times 10^{-3}\,M$). The optimum concentration of glutathione and of ascorbate was found at $1.3 \times 10^{-3} M$ in both cases.

In Fig. 5 are shown the results of the experiments in which the activation of the Fe^{++}-free enzyme brought about by glutathione and ascorbic acid in the presence of ferrous ion was compared at various pH values. It will be seen that the pH-dependency of the activation caused by glutathione was quite similar to that caused by ascorbate in that both agents had the highest activation effect at about pH 7.0 and they were rather inhibitory at acid pH's.

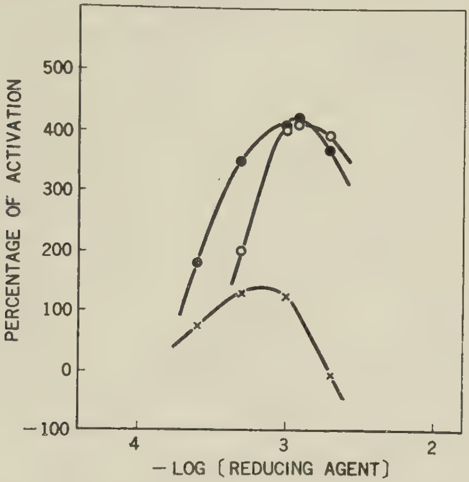

FIG. 4. Activation caused by reducing agents in various concentrations.

Activity assay was made by manometric method at 30°, pH 7.0. Each vessel contained the Fe^{++}-free enzyme solution (1.7 mg. protein), $5 \times 10^{-4} M$ $FeSO_4$, $3.3 \times 10^{-3} M$ of homogentisic acid, $4 \times 10^{-2} M$ phosphate buffer, reducing agent and distilled water to make the final volume 2 ml. In the control system, the reducing agent was omitted. Percentage of activation was expressed as $(V/V_0\text{-}1) \times 100$, where V is the reaction velocity of the reducing agent containing system and V_0, that of the control system.

● : value obtained in presence of glutathione.
○ : value obtained in presence of ascorbic acid.
× : value obtained in presence of cystein.

In order to test whether or not these effects of glutathione and ascorbate are ascribable to the reduction of the same specific site(s) of the enzyme protein, the following experiments have been carried out. The rate of reaction of the system, in which both glutathione and ascorbate were simultaneously added at their respective optimal concentrations, was compared with the rates of the control systems composed of one of these reagents. Fig. 3 also shows that glutathione could not activate the reaction of the system, in which a sufficient amount of ascorbate existed, and *vice versa*. These results are incompatible with Crandall's claim (*2*) that glutathione exerts an additional activation effect to that carried by ascorbate.

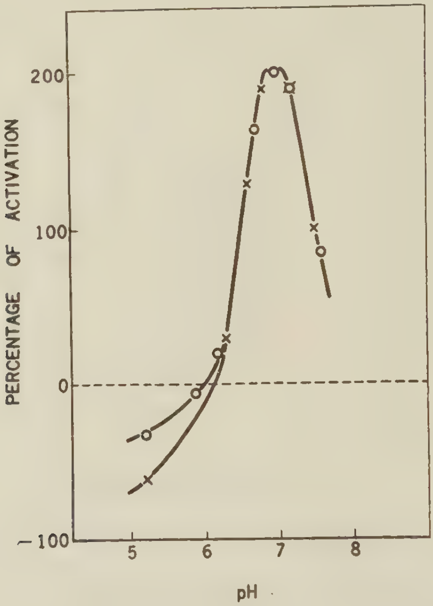

FIG. 5. Relationships between pH and activation by glutathione and ascorbic acid.

Each vessel contained Fe^{++}-free enzyme solution (2.3 mg. protein), $5\times10^{-4}\,M$ of $FeSO_4$, $3.3\times10^{-3}\,M$ of homogentisic acid, $4\times10^{-2}\,M$ of phosphate buffer, $1\times10^{-3}\,M$ of glutathione or ascorbic acid and distilled water to make the final volume of 2 ml. All experiments were carried out manometrically at 30°. In the test system, both ferrous ion and reducing agent were incubated for 30 minutes with buffered enzyme solution at given pH, 30°, and in the control system, in which reducing agent was omitted, ferrous ion alone was previously incubated with the enzyme solution under the same condition as described above. Reaction was initiated by adding the substrate. Percentage of activation was estimated according to the formula $(V/V_0\text{-}1)\times100$, where V and V_0 represent the values of oxygen consumption of the initial 10 minutes at the same pH in the presence and absence of reducing agent, respectively.

× : value obtained by adding glutathione.

○ : value obtained by adding ascorbic acid.

It may be concluded from these results that both reagents react with the same site(s) of the enzyme, and that their activating effects are due to the maintenance of a certain essential group(s) of the enzyme in some reduced state.

Since the possibility still remained that these reagents might exert their activating effects by maintaining the iron attached to the enzyme in the ferrous state, the effect of sodium borohydride on the enzymatic activity was studied. As may be seen in Fig. 6, no activation was effected by borohydride on the enzymatic reaction although the reagent reduced iron from the ferric to the ferrous state with considerable velocity. The Fe^{++}-free enzyme could not be activated by the addition of both ferric ion and glutathione (data are

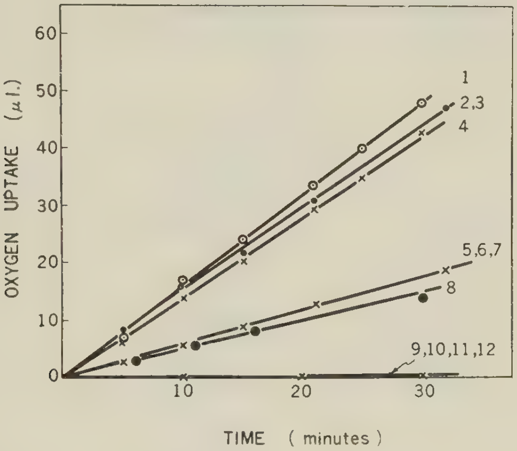

FIG. 6. Comparison of effects of glutathione and borohydride.

Enzymatic activity was measured manometrically at 30°, pH 6.6. Gas phase was air. Each vessel contained 2.15 mg. of Fe^{++}-free enzyme preparation, $2\times10^{-3}\,M$ of homogentisic acid, $4\times10^{-2}\,M$ of phosphate buffer and distilled water. The following additions were made to each system. Total volume was 2 ml.

1. $FeSO_4$ ($1\times10^{-3}\,M$)+glutathione ($1.3\times10^{-3}\,M$)
2. $FeSO_4$ ($1\times10^{-3}\,M$)+glutathione ($1.3\times10^{-3}\,M$) +borohydride ($4\times10^{-3}\,M$)
3. $FeSO_4$ ($1\times10^{-3}\,M$)+glutathione ($2\times10^{-3}\,M$)
4. $FeSO_4$ ($1\times10^{-3}\,M$)+glutathione ($2\times10^{-3}\,M$) +borodydride ($4\times10^{-3}\,M$)
5. $FeSO_4$ ($1\times10^{-3}\,M$)+borohydride ($4\times10^{-3}\,M$)
6. $FeSO_4$ ($1\times10^{-3}\,M$)+borohydride ($2\times10^{-3}\,M$)
7. $FeSO_4$ ($1\times10^{-3}\,M$)
8. $FeSO_4$ ($1\times10^{-3}\,M$)+borohydride ($8\times10^{-3}\,M$)
9. Glutathione ($1.3\times10^{-3}M$)+borohydride ($4\times10^{-3}M$)
10. Glutathione ($1.3\times10^{-3}\,M$)
11. Borohydride ($4\times10^{-3}\,M$)
12. None

not shown) although it was activated by the addition of ferric ion and ascorbate as reported by Suda and Takeda (*12*).

Therefore, it does not seem likely that activation by glutathione and ascorbate is due to the maintenance of a certain level of ferrous ion by their reducing power. Instead, it appears more reasonable to assume that these effective reducing agents act on the active SH group of the enzyme and maintain it in the reduced form.

Effects of SH Reagents—It confirmed the work of other investigators (*2*, *4*), as indicated in Table I and II, that the activity of homogentisicase is strongly inhibited by various SH reagents such as *p*-chloromercuribenzoate (PCBM), mono-iodoacetate, ferricyanide, *o*-iodosobenzoate and mercuric chloride. It is also shown in Table II that these inhibitions could be markedly

TABLE I

Inhibition by SH Reagents

Compound	Concentration (M)	Inhibition (%)
p-Chloromercuribenzoate	4×10^{-4}	100
	2×10^{-4}	85
	1×10^{-4}	82
Monoiodoacetate	4×10^{-3}	43
	2×10^{-3}	37
	1×10^{-3}	22
	5×10^{-4}	21
Ferricyanide	2×10^{-3}	100
	1×10^{-3}	90
	5×10^{-4}	85
	2.5×10^{-4}	76

Assay was made manometrically at pH 7.0, 30°. Gas phase was air. Each vessel contained the Fe^{++}-free enzyme solution (3.35 mg. protein), $5\times10^{-4}\,M$ of $FeSO_4$, $3.3\times10^{-3}\,M$ of homogentisic acid, $4\times10^{-2}\,M$ of phosphate buffer, SH reagent and distilled water to make the final volume 2 ml. In the control system, the inhibitor was omitted. Inhibitor was preincubated with the enzyme solution in phosphate buffer for 15 minutes before the addition of both ferrous ion and the substrate. Inhibition was expressed by $(1-V_i/V_o)\times100$, where V_i and V_o are the reaction velocities of the systems in presence and absece of the inhibitor, respectively.

prevented when the enzyme was preincubated with the substrate in the presence or in the absence of the inhibitors. It is especially remarkable to note that inhibition caused by PCBM, which is the most powerful and specific reagent for SH groups, was completely destroyed by preincubation with the substrate. The enzyme was, however, not protected from these reagents by the presence of ferrous ion during preincubation. The experiments given in Table II were those conducted at pH 6.8; essentially the same results were also obtained at pH 6.0 and 7.2. These facts suggested that the substrate combined with a certain SH group or groups of the enzyme and thus pro-

Table II

Inhibition SH Reagents at Various Conditions[1]

Exp. No.	Inhibitor	Preincubation[2]	Subsequent incubation[3]	Degree of inhibition (%)
1.	PCMB (4×10^{-5} *M*)	E+PCMB	$+Fe^{++}+S$	100
		$E+PCMB+Fe^{++}$	+S	85
		E+PCMB+S[4]	$+Fe^{++}$	44
		$E+Fe^{++}$	+PCMB+S	70
		E+S[4]	$+PCMB+Fe^{++}$	0
2.	*o*-Iodosobenzoate (IBA) (2×10^{-3} M)	E+IBA	$+Fe^{++}+S$	100
		$E+IBA+Fe^{++}$	+S	98
		E+IBA+S[4]	$+Fe^{++}$	68
		$E+Fe^{++}$	+IBA+S	95
		E+S[4]	$+IBA+Fe^{++}$	53
3.	Monoiodoacetic aicd (IAA) (1×10^{-2} *M*)	E+IAA	$+Fe^{++}+S$	93
		$E+IA+Fe^{++}$	+S	94
		E+IA+S[4]	$+Fe^{++}$	57
		$E+Fe^{++}$	+IAA+S	80
		E+S[4]	$+IAA+Fe^{++}$	45
4.	$HgCl_2$ (3.3×10^{-4} *M*)	$E+HgCl_2$	$+Fe^{++}+S$	100
		$E+Fe^{++}$	$+HgCl_2+S$	100
		E+S[4]	$+HgCl_2+Fe^{++}$	0

1) Symbols used in the table have the following denotations: E; Fe^{++}-free enzyme solution, S; homogentisic acid.

2) Systems were preincubated for 15 minutes.

3) Reaction was initiated by the additon of the components indicated.

4) Degree of inhibition increased after initial 5 minutes.

Assay was made manometrically by measurement of O_2 uptake at initial 5 minutes. Each vessel contained the Fe^{++}-free enzyme solution (No. 1; 2.8 mg., No. 2; 4 mg., No. 3; 2.5 mg., and No. 4; 1.75 mg. protein, respectively), 1×10^{-3} *M* $FeSO_4$, 3.3×10^{-3} *M* homogentisic acid, 4×10^{-2} *M* phosphate buffer, the SH reagent, and distilled water to make the final volume 2 ml. Gas phase was air. Temperature: 30°, pH: 6.8. The control systems were also treated with the same procedure, except no addition of inhibitor. The degree of inhibition was expressed by $(1-V_i/V_0)\times100$.

tected it from the action of SH reagents.

It was also observed that when PCMB ($2.5\times10^{-4}\,M$) was preincubated with the Fe^{++}-enzyme (5 mg. protein) at 30° for 15 minutes in phosphate buffer ($4\times10^{-2}\,M$) and then the reaction was started by adding simultaneously the substrate ($3.3\times10^{-3}\,M$) and ferrous ion ($5\times10^{-4}\,M$), the degree of inhibition was about the same (40 per cent) over a wide pH-range (pH 7.0, 6.7, 6.2 and 5.0). It seems likely, therefore, that the dissociation group of the enzyme which participates in the combination of ferrous ion is not attributable to the SH groups of the enzyme protein.

It was further tested by the following experiment whether ferrous ion can be released from the Fe^{++}-enzyme by treatment with PCMB. The Fe^{++}-enzyme (10 mg. protein) was mixed with an excess of PCMB ($10^{-3}\,M$) at pH 7.0. After incubating the mixture at 30° for several minutes, the substrate ($1.7\times10^{-3}\,M$) was added to it to confirm complete inhibition. The completely inhibited enzyme was precipitated from the mixture by adding solid ammonium sulfate to 0.85 saturation, and the concentration of ferrous ion in the supernatant was measured by the *o*-phenanthroline method. It was

TABLE III

*Activity of Enzyme Treated with Tyrosinase*1)

Exp. No.	Preincubation2)	Time of preincubation (*min.*)	Subsequent incubation3)	O_2 uptake (*µl./10 min.*)	Inhibition (%)
1	Fe^{++}-enzyme	40	+S	30	
	Fe^{++}-enzyme+ tyrosinase	40	+S	30	0
2	Fe^{++}-free enzyme	40	$+Fe^{++}+S$	44	
	Fe^{++}-free enzyme+ tyrosinase	10	$+Fe^{++}+S$	25	44
	,,	20	$+Fe^{++}+S$	18	59
	,,	40	$+Fe^{++}+S$	10	78
3	Fe^{++}-free enzyme	25	$+Fe^{++}+S$	14	
	Fe^{++}-free enzyme+ tyrosinase	25	$+Fe^{++}+S$	2	86
	Fe^{++}-free enzyme+ tyrosinase+S	25	$+Fe^{++}$	14	0

1) Symbol S described in the second and the fourth columns means homogentisic acid.

2) Systems were preincubated at 30° before the assay.

3) Assay was initiated by addition of the components described.

Each vessel contained the enzyme solution (No. 1; 3.1 mg., No. 2; 5.7 mg., No. 3; 2.3 mg. protein, respectively), $5\times10^{-4}\,M$ $FeSO_4$, $3.3\times10^{-3}\,M$ homogentisic acid, $4\times10^{-2}\,M$ phosphate buffer, and distilled water to make the final volume 2 ml. In the case of tyrosinase-containing system, the tyrosinase solution (360 µg. protein) was placed instead of distilled water and its specific activity was expressed as the value of 150 µl. O_2/5 min./mg. protein. All experiments were carried out by manometric method at 30°, pH 6.6. Gas phase was air.

thus found that the ferrous-phenanthroline color developed in the supernatant was negligibly weak and it was almost the same as that developed in the control experiment in which PCMB was not added (optical density at 500 mμ= 0.025). From this result, which indicates that PCMB can not release the ferrous ion from the enzyme protein, it seems probable that the SH group of the enzyme does not bind with ferrous ion but it does so with the substrate.

Inhibition by Tyrosinase—Since Sizer (*13*) first showed that tyrosinase can inactivate several enzymes by its rather unusual capacity for oxidizing tyrosine residues in a protein, this enzyme has frequently been used as a tool for the study of the role of phenolic hydroxyl groups in the enzymatic catalysis. It has been reported (*14*) that the Fe^{++}-free preparation of homogentisicase is susceptible to the action of tyrosinase. In view of the importance of this finding, it has been decided in the present study to reexamine the effect of tyrosinase in some detail. The tyrosinase preparation employed was completely inactive to homogentisic acid and its tyrosine-oxidizing activity was unaffected by the addition of either ferrous ion (10^{-3} *M*) or homogentisic acid (3×10^{-3} *M*). As can be seen from the results recorded in Table III, it was found that Fe^{++}-enzyme is quite resistant to the action of tyrosinase. The activity of the Fe^{++}-free enzyme, on the other hand, was markedly inhibited by the treatment with tyrosinase, and the degree of inhibition increased with increasing preincubation period. It was, however, revealed that the presence homogentisic acid during preincubation completely protected the Fe^{++}-free enzyme from attack by tyrosinase. In contrast to a previous report (*14*), indicating a protective effect of ferrous ion against the tyrosinase-catalysed inactivation of the Fe^{++}-free enzyme, it was not always possible, in the present study, to demonstrate such protective effect.

Although it appears still premature to draw a decisive conclusion from the observations described above, it seems probable that the phenolic hydroxyl groups of the enzyme protein are in some way involved in the catalytic activity of homogentisicase.

DISCUSSION

The experimental results reported in the present paper suggest that homogentisicase possesses at least two types of reactive groups, namely the SH group which has also been recognized by other workers (*2*, *4*) and the phenolic group which is susceptible to the action of tyrosinase.

The observations that glutathione and ascorbic acid not only activate the Fe^{++}-enzyme but also enhance activation by ferrous ion of the Fe^{++}-free enzyme may be accounted for by assuming that these reducing agents exert their actions by keeping the SH group of the enzyme protein in the active, reduced state. A similar explanation may also apply to the effect of these agents in protecting the enzyme from inactivation during storage (*1*). It is noted that the degree of activation caused by these reducing agents considerably varies from one enzyme preparation to the other. This suggests that the enzyme is reversibly inactivated to various degrees by the oxidation of its essential SH group in the course of purification. Although it has sometimes been claimed that glutathione is a coenzyme of homogentisicase (*14*), it has not been possible to detect any specificity in the activation effects of glutathione and ascorbic acid. Therefore, it does not seem likely that glutathione is a specific cofactor or coenzyme in the usual sense of the term.

The possibility that these reducing agents activate the enzyme by maintaining the level of essential ferrous ion in the reduced state seems to be less likely in view of the fact that the enzyme is not activated by sodium borohydride although this reagent reduces ferric iron to ferrous iron with considerable velocity. The borohydride has also been found to be unable to reduce the -S-S- group of cystine to the SH state under the conditions employed in this study. It is shown in the preceding paper (*1*) that the essential iron is always kept in the ferrous state during the enzymatic reaction under aerobic conditions.

Knox (*4*) and Crandall (*2*) have claimed that homogentisicase is the first example of "iron-sulfhydryl enzyme" and suggested that the essential iron combines with the enzyme through its SH group. This suggestion is, however, incompatible with the observations described in the present paper. In the present study it was shown that the inhibition by SH reagents could be reversed by preincubating the enzyme with the substrate, but preincubation with ferrous ion was completely ineffective in preventing inhibition. The fact that the treatment of the Fe^{++}-enzyme with PCMB does not expel the bound ferrous ion from the enzyme protein is also difficult to explain on the basis of the suggestion made by Knox and Crandall. A more plausible explanation, consistent with all the data obtained in the present investigation, is that the SH group is the site at which the substrate combines.

The involvement of the phenolic group in the enzymatic activity is clearly demonstrated by the inhibition caused by the treatment of the Fe^{++}-free enzyme with tyrosinase. There is, however, some ambiguity as to the role of the phenolic group, since it is shown, on one hand, that the Fe^{++}-enzyme is resistant to the action of tyrosinase, and, on the other hand, that preincubation of the Fe^{++}-free enzyme in the presence of the substrate markedly protects the enzyme from attack by tyrosinase. It seems probable that both the substrate and ferrous ion interact with the phenolic group in some complicated manner. Although no direct evidence could be obtained, the essential phenolic group(s) of native enzyme seems to be embeded in the enzyme protein and to appear on its surface by partial denaturation or unfolding caused by the removal of the ferrous ion. Such phenolic group(s) which appears on the surface of enzyme may be oxidized by treating with tyrosinase. The conversion to native or folded form seems to occur by the combination of the substrate or ferrous ion with the enzyme protein.

Little information can be obtained from the results described in the present paper concerning the mode of attachment of the essential ferrous ion. The behavior of the ferrous ion as reported in the preceding paper (*1*) and the kinetic studies to be described in a following paper (*15*), however, seem to suggest that the ferrous ion combines with a basic group having a pK similar to that of the imidazole group. There is no evidence indicating that the ferrous ion intervenes as a single bridge former.

Finally, it is interesting to note that the properties and behavior of homogentisicase are very similar to those of pyrocatechase (*16*, *17*). It is quite possible that the two enzymes have similar reaction mechanisms, especially with respect to the action of ferrous ion as their cofactor.

SUMMARY

1. The stoichiometry of the reaction catalysed by homogentisicase was confirmed. It was in accordance with the equation;

$$\text{Homogentisic acid} + O_2 = \text{maleylacetic acid}$$

2. Effects of glutathione and ascorbic acid on the homogentisicase activity were studied. Based on these studies it was concluded that the activation of the enzyme by these reducing agents was due to the maintenance of the essential SH group in the reduced state.

3. Inhibition caused by various SH reagents was investigated in some detail. It was found that inhibition can be markedly reversed by preincubating the enzyme with the substrate. Ferrous ion failed to protect the enzyme from SH inhibitors. The enzyme-bound ferrous ion could not be released by the treatment of the enzyme with PCMB. From these observations it was concluded that the SH group is the site of the substrate combination.

4. It was shown that the Fe^{++}-free enzyme was inactivated by the action of tyrosinase, and that inactivation could be prevented by the presence of the substrate. The Fe^{++}-enzyme was found to be stable against the action of tyrosinase even in the absence of the substrate. These observations were taken as evidence indicating that phenolic group was involved in the enzymatic activity.

The author wishes to express her deep gratitude to Prof. M. Suda for his kind guidance and to Prof. H. Tamiya, Dr. Y. Ogura and Dr. R. Sato for their valuable advice and discussion.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

RNA DEGRADATION AND DNA AND PROTEIN SYNTHESIS OF E. COLI B. IN A PHOSPHATE DEFICIENT MEDIUM*

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(Received for publication, April 6, 1959)

It has been reported by several authors (*e.g.* Halvorson (*1*)) that the turnover rate of nucleic acid and protein is quite different between growing and resting microorganisms. However, the cellular response would naturally show a wide variation depending upon the condition under which organisms are led into resting stage.

The present paper is concerned with a degradation of RNA** and its relevant phenomena in *E. coli* B. under the phosphate deficient state. Among them a formation of alkaline phosphomonoesterase (PMase) which was repressed by phosphate was remarkable.

A preliminary report of this paper has already been published (*2*).

EXPERIMENTALS

Strain—The strain used in all experiments was *Escherichia coli* strain B.

Reagents—*o*-Nitrophenyl-β-D-galactoside and bis-*p*-nitrophenyl phosphate were kindly supplied by Drs. D. Mizuno and A. Ohsaka in our laboratory. Crystalline samples of chloramphenicol and disodium *p*-nitrophenyl phosphate were obtained from the Sankyo Co., Ltd. and Daiichi Pure Chemicals Co., Ltd., respectively. Purified yeast RNA (Wako Pure Chemical Industries, Ltd.) was dissolved in 0.05 *M* acetate buffer (pH 5.0) and dialyzed for 48 hours against demineralized water at 4°. The water was frequently changed. Then the dialysed solution was filtered and lyophilized. In the estimation of enzyme activity to be reported, this lyophilized RNA was used as substrate. A carrier free radioactive phosphate was supplied by Japan Radioisotope Association (originally furnished by the Radiochemical Center, Amersham, England).

Media -Peptone medium contained per liter: 3.0 g. NaCl, 0.25 g. $MgSO_4 \cdot 7H_2O$, 0.01 g. $CaCl_2$, 2.0 g. sodium lactate, 10.0 g Difco Bactopeptone brought to pH 7.4 with NaOH and sterilized at 100° three times. This medium contained 16 μg. P/ml.. In a large part of experiments, one twentieth volume of strerilized 1 *M* Tris buffer (pH 7.4) was added to the one volume of the peptone medium.

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* This work was supported in parts by a grant supplied from the Ministry of Eduction of Japan and Hoansha Foundation.

** The following abbreviations will be used: ribonucleic acid (RNA), desoxyribonucleic acid (DNA), optical density (OD), tris-(hydroxymethyl)-aminomethane (Tris), trichloroacetic acid (TCA), alkaline phosphomonoesterase (PMase), phosphodiesterase (PDase), ribonuclease (RNase).

Peptone-Phosphate Medium -One three hundredth volume of sterilized 1 *M* phosphate buffer (pH 7.4) was added to one volume of the peptone medium. This medium contained about 120 μg. P/ml..

Measurement of Bacterial Growth—Bacterial growth was measured by Coleman spectrophotometer and expressed in terms of the optical density (OD) at a wave length of 650 mμ (light path : 1.9 cm). In some cases viable counts of the cells were used.

Cultural Conditions—Cells were inoculated from a slant to the peptone medium and keep at 37° for several hours with aeration by shaking. The culture showing the OD about 0.5 was used as a seed culture. The experiment was started by the inoculation of seed culture (one twentieth of the volume) to the peptone medium. The phosphate in the medium is exhausted normally 2.5–3 hours after the inoculation. At this time, the OD of the culture is about 0.30–0.35 at a 650 mμ. This time will be referred to as " phosphate exhaustion time* " in this report. The OD continue to increase linearly to the value of about 0.65 for 3–4 hours after the phosphate exhaustion time. This phase will be referred to as " phosphate deficient phase ". The " phosphate deficient bacteria " in this report means the cells present at the last stage of the phosphate deficient phase. When the seed culture was inoculated in the peptone-phosphate medium, OD showed about 0.40 for 3 hours incubation thereafter. The " growing bacteria " herein described means the cells present at this phase.

Zone Electrophoresis—Cells free extracts were obtained by the following procedure. Cells were harvested, washed with Tris buffer (pH 7.8, ionic strength=0.02), ground with 2.5–3 times their wet weight of almina in the cold at 4° (*3*), and suspended in a small amount of Tris buffer. Then the suspension of the cell extract was centrifuged at 5,000×g for 5 minutes at less than 4° to remove intact cells, cell debris and almina. In some cases, washed cells were suspended in a small volume of Tris buffer, and exposed for 10 minutes in the 20 KC. sonic oscillator (Toyo Riko, Type 50–4).

Starch electrophoresis was performed by the method similar to those described by Pardee *et al.* (*4*). The commercial potato starch was washed with a large volume of water until substances showing the spectra at 280 mμ in washings were completely removed, being followed finally by several times of washings with the Tris buffer solution (pH 7.8, ionic strength=0.02) to be employed. This starch preparation was used as the supporting medium. A transverse hole was cut in the starch about 10 cm from the cathode and this was filled with 1.5 ml. of bacterial extract mixed with 2.4 g. dried starch. To obtain the simultaneous analysis of 2 samples under identical conditsons, the runs were carried out with two troughs (1.0×2.5×45.0 cm. each) set pararelly. One trough contained the extract of growing cells, and the other contained the extract of phosphate deficient cells. A current of 2.5 mA. was maintained by a potential drop of 125 volts and the experiment was allowed to run for 20 hours at about 4°. The starch block was then cut crosswise into sections 1 cm wide, each of which was extracted with 5 ml. of demineralized ice cold water**.

Ultracentrifugal Analysis—Cell free extracts were obtained by the method described in zone electrophoresis. Instead of Tris buffer, 0.02 *M* NaCl solution was used (*5*).

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* There is an initial phase of rapid growth followed by a phase of slow growth. We tentatively call the time when the growth rate changed as " phosphate exhaustion time ".

** The author is indebted to the staffs of Dr. I. Watanabe's laboratory (Department of Biochemistry, Faculty of Science, University of Tokyo) for their interests and helpful discussions.

Ultracentrifugal analysis of the extracts were conducted in a Spinco ultracentrifuge Model E* .

Chemical Determinations—Aliquots of whole bacterial culture were precipitated with cold 0.3 M trichloroacetic acid (TCA). The precipitated DNA was estimated by the diphenylamine method (*6*) using calf thymus DNA as a standard. The precipitated RNA was estimated by orcinol reaction (*7*) using yeast RNA as a standard. The estimation of protein nitrogen by Nessler's method (*8*) have been described by Tomizawa and Sunakawa (*9*). In some cases, hot-TCA insoluble protein was determined by the modification (*10*) of Folin's method, using bovine serum albumin as a standard.

Determination of Enzyme Activities—Aliquots of cell culture were sampled, chilled in an ice bath, and washed twice with an ice cold physiological saline. Then the (centrifuged) cells were resuspended in the original volume of ice cold physiological saline. These cell suspensions were added 3–5 *v/v* per cent toluene and incubated at 37° for 10 minutes. In the case of the estimation of PMase, PDase and β-galactosidase, 10 μg./ml. of sodium desoxycholate and 50 μg./ml. of chloramphenicol were added with toluene. Then the suspensions were shaken vigorously and kept at 37° for additional 20 minutes (*11*). These toluene treated cell extracts were used in the determination of enzyme activities. These extracts showed equal activities to those obtained by sonic oscillation.

Estimation of Alkaline Phosphomonoesterase (*PMase*)—The principle of the assay was similar to the method of Bessey *et al.* (*12*). Reaction mixture contained 0.12 M glycine-NaOH buffer (pH 9.5), $0.9\times10^{-3}\,M$ *p*-nitrophenyl phosphate, $2.7\times10^{-2}\,M$ $MgSO_4$ and an extract of toluene-lysed bacteria in 5.5 ml.. The reaction mixture was incubated at 37°, and the reaction was halted with the addition of 1 ml. of 1 N NaOH at 15 minutes. Then samples were cooled by tap water and centrifuged. Enzyme activity was assayed by the colour development of *p*-nitrophenol splitted with Coleman spectrophotometer at the wave length of 420 mμ.

Estimation of Phosphodiesterase (*PDase*)—The principle of the assay was similar to the method of Yoshida (*13*). Reaction mixtures contained 0.04 M Tris buffer (pH 7.5), bis-*p*-nitrophenyl phosphate $0.8\times10^{-4}\,M$, $CoCl_2$ $1.6\times10^{-4}\,M$ and an extract of bacteria in 2.5 ml.. The reaction mixture was incubated at 37° and the reaction was halted with the addition of 2 ml. of 0.3 N NaOH at 30 minutes. Enzyme activity was assayed by colour development of *p*-nitrophenol splitted with Coleman spectrophotometer at the wave length of 420 mμ.

Estimation of β-Galactosidase—The principle of the assay was similar to the method of Lederberg (*14*). Reaction mixture contained 0.06 M phosphate buffer (pH 7.4), $5\times10^{-4}\,M$ *o*-nitrophenyl-β-D-galactoside and an extract of bacteria in 10 ml.. The reaction mixture was incubated at 37° and the reaction was halted with the addition of 1 ml. of 1 M Na_2CO_3 at 20 minutes. Enzyme activity was assayed by the colour development of *o*-nitrophenol splitted with Coleman spectrophotometer at the wave length of 420 mμ.

Estimation of Ribonuclease (*RNase*)—The principle of the assay was similar to that described by McDonald (*15*). Reaction mixtures contained 0.1 M Tris buffer (pH 7.5), RNA 2.5 mg./ml. and an extract of bacteria in 2 ml. The reaction mixture was incubated at 37° and reaction was halted by the addition of 2 ml. of uranyl TCA (uranyl acetate 0.25 per cent and TCA 2.5 per cent) at 30 minutes, then mixtures were kept for 30 minutes at 37° and centrifuged at 3,000 r.p.m. for 10 minutes. The supernatant were separated carefully from the precipitate and diluted with 4 volumes of demineralized water. Enzyme activity was assayed by the development of absorbance at the wave length of 260 mμ in the final samples with Beckman Model DU spectrophotometer.

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* The author is indebted to Dr. S. Kubota and Miss M. Takahashi (Department of Pharmaceutical Sciences, University of Tokyo) for taking the photographs.

Radioisotopical Determinations—Medium fraction: the supernatant of the whole culture obtained by centrifugation at 5,000×*g* for 5 minutes. *Acid soluble fraction*: the extract of washed cells with ice-cold 5 per cent TCA for 1 hour. *RNA and DNA fraction*: RNA-P^{32} and DNA-P^{32} were obtained by Hershey's procedure (*16*) of Schmidt and Thannhauser's method (*17*) with the addition of processes of lipide elimination by ether-alcohol (3 : 1) and alcohol.

RESULTS

RNA Degradation in the Phosphate Deficient Phase—As illustrated in Fig. 1 (A), the amount of P^{32} incorporated into the RNA per ml. in poor phosphate medium (peptone medium) reached its maximal level when the inorganic phosphate was exhausted, and, thereafter, decreased by 20–30 per cent during the subsequent phosphate deficient phase. On the other hand, during this phase, P^{32} was incorporated into DNA fraction gradually, and a slight increase of acid soluble-P^{32} and of medium-P^{32} was observed. The amount of the RNA and of DNA estimated by orcinol and diphenylamine, respectively, (Fig. 3) and ultraviolet absorption of these fractions obtained by the method of Schmidt and Thannhauser, showed similar results. A slowed but constant rate of OD increment was observed in this stage. The amount of protein was shown quite proportional to the OD (Fig. 6).

Since the RNA in the final stage of phosphate deficient phase seems to

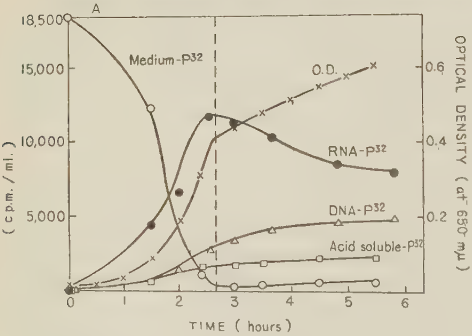

FIG. 1 (A). Degradation of (P^{32}) RNA in the phosphate deficient medium.

The seed culture of *E. coli* B. was suspended in a peptone medium containing carrier-free P^{32} (final concentration, 18,500 c.p.m./ml.) at zero time and incubated at 37° with aeration by shaking. The broken line indicates the time when the phosphate in the medium is exhausted.

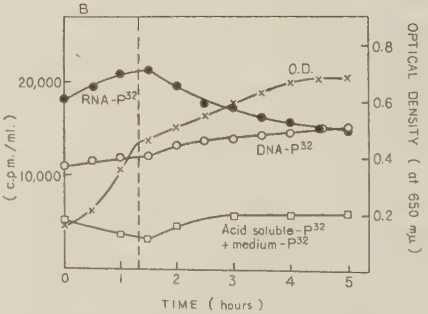

FIG. 1 (B). Secondery degradation of (P^{32}) RNA remaining after exhaustion of the inorganic phosphate.

Cells harvested from a P^{32}-containing culture 3–4 hours after exhaustion of the inorganic phosphate were thoroughly washed and resuspended in a P^{32}-free medium to follow the zero time. P^{32} radioactivity of cell suspension at zero time was 44,000 c.p.m./ml..

be stable, the stability of this RNA was tested. Phosphate deficient cells harvested from a P^{32}-containing culture 3–4 hours after phosphate exhaustion time were thoroughly washed and resuspended in a P^{32}-free peptone medium. As shown in Fig. 1 (B), RNA-P^{32} increased to a considerable extent at the expense of the acid soluble-P^{32} until the phosphate in the medium was exhausted. But the degradation of RNA was again observed in this case during the phosphate deficient phase although DNA-P^{32} increased gradually. The amount of the DNA-P^{32} was almost equal to that of RNA-P^{32} at the end of the phosphate deficient phase.

These results indicate the following alternatives: the RNA which would have been stable became partially unstable after the exposure to the normal growing stage, or that the RNA is not essentially stable.

The stability of the RNA may possibly vary depending upon whether it is synthesized in the early or late logarithmic phase. Contrary to the expectation, as illustrated in Fig. 1 (C) and (D), both of the RNA-P^{32} start to decrease almost concomitantly with the exhaustion of the phosphate and 20–30 per cent of RNA per ml. degraded at the end of the phosphate deficient phase.

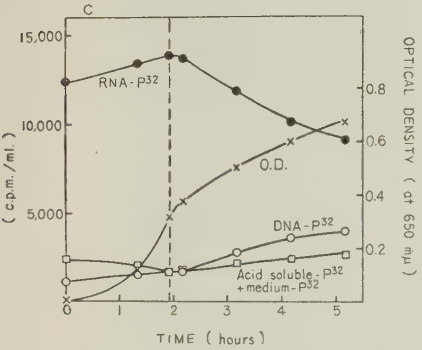

FIG. 1 (C). Degradation of (P^{32}) RNA synthesized in the early logarithmic phase. The seed culture was suspended in a P^{32}-containing peptone medium and kept incubated at 37° for 30 minutes. Cells were harvested, washed twice, and resuspended in a peptone medium. P^{32} radioactivity of the cell suspension at zero time was 19,000 c.p.m./ml..

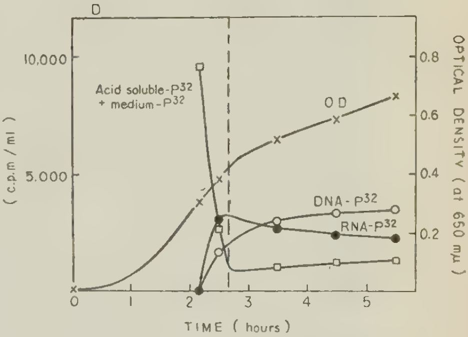

FIG. 1 (D). Degradation of (P^{32}) RNA synthesized in the later logarithmic phase. The seed culture was incubated in a peptone medium at zero time and carrierfree P^{32} was added to the culture at 130 minute (9,500 c.p.m./ml.).

Since the P^{32} in acid soluble fraction and in the medium does not show so much increase, these data suggest that a large portion of the P^{32} released from RNA fraction was utilized for the synthesis of DNA after the phosphate exhaustion time.

Ultracentrifugal and Electrophoretical Patterns of Cell Free Extracts from the Growing and the Phosphate Deficient Bacteria—As reported previously (*2*) in ultracentrifugal pattern, the ratio amount of the larger particles (40 *s* and 29 *s*) to that of the smaller ones (8 *s* and 5 *s*) was found to be much lower in the phosphate deficient than in the growing cells. Therefore, the degradation of 40 *s* and 29 *s* particle fraction constituted mainly of RNA and protein (*5*) may be possible.

Pardee *et al.* (*4*) reported that the RNA in *E. coli* extract can be separated into two fractions, large particle nucleoprotein containing above mentioned 40 *s* and 29 *s* particles and low molecular weight RNA, by the use of zone electrophoresis.

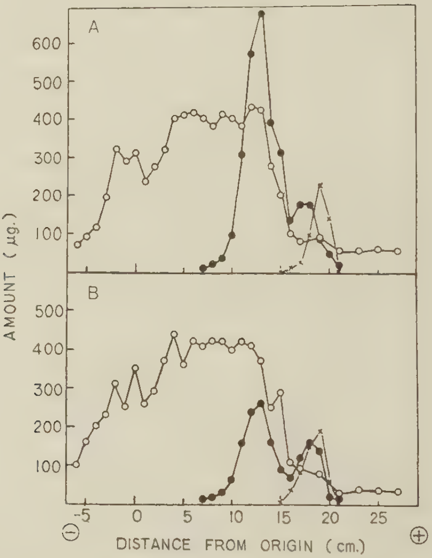

FIG. 2. Starch electrophoretical patterns of extracts prepared by almina grinding of *E. coli* B.

(A) Growing bacteria (see Experimentals)

(B) Phosphate deficient bacteria (see Experimentals)

The μg. of materials found at various positions are depicted on the ordinate. —○— protein (×1/1.5), —●— RNA, —×— DNA.

As depicted in Fig. 2, two fractions of RNA were obtained electrophoretically in the extract of growing cells as well as that of phosphate deficient cells. Results obtained by several experiments showed that the ratio of the amount of the large particle RNA to that of low molecular weight RNA was about 8–5 to 1 in the case growing cells and 3.5–2 to 1 in the case of phosphate deficient cells. Precise comparison concerning the ratio of amounts of the two RNA fractions is not possible in these experiments, but it will be evident that the decrease of the amount of the large particle RNA was found to be larger than that of the low molecular weight RNA in the phosphate deficient cells comparing with the growing cells.

The Effects of Several Drugs on RNA Degradation, DNA Synthesis and OD Increment—2, 4-dinitrophenol (final concentration; 2×10^{-3} *M*), NaN_3 (5×10^{-3} *M*), Na_2HAsO_4 (5×10^{-3} *M*) and chloramphenicol (1×10^{-4} *M*) were added to the early phosphate deficient phase culture.

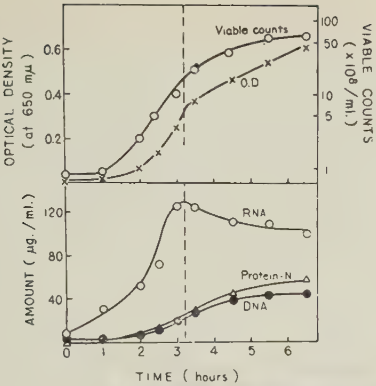

FIG. 3. Protein and nucleic acid contents of phosphate deficient cells.

The seed culture of *E. coli* B. was suspended in a peptone medium at zero time and incubated at 37° with aeration by shaking. Viable counts were plotted in a logarithmical scale. The broken line indicates the time when the phosphate in the medium should be exhausted.

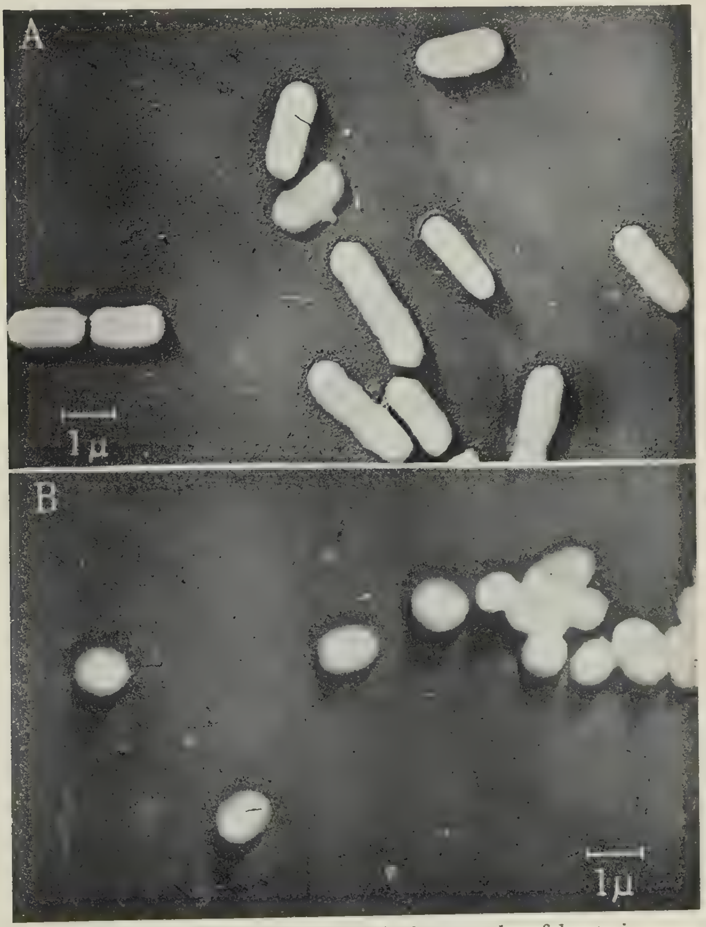

FIG. 4. Electron microscopical photographs of bacteria.
A : growing bacteria (see Experimentals).
B : phosphate deficient bacteria (See Experimentals).
(Courtesy of Dr. S. Nakazawa in our laboratory.)

2, 4-dinitrophenol inhibited DNA synthesis and OD increment completely, but the effect of this drug on RNA degradation was variable. Na_2HAsO_4 also inhibited DNA synthesis and OD increment completely. About double the amount of the control RNA degradation was observed in the presence of Na_2HAsO_4 during 3 hours. But the mode of its degradation is linear against the time being quite different from the control. NaN_3 exerted little effect on them. In the presence of chloramphenicol the RNA was conserved, whereas the DNA and OD increased slightly.

Protein and Nucleic Acid Contents of Phosphate Deficient Bacteria—During the phosphate deficient phase, the amounts of DNA (estimated by Dische's reaction (*6*)) and protein nitrogen (estimated by Nessler's method (*8*)) doubled despite a 20–30 per cent decrease of RNA (estimated by orcinol reaction (*7*)). But the increment of bacterial counts was more marked amounting to a 5–6 fold increase (Fig. 3). It is, therefore, evident that during this stage the contents of nucleic acids and protein per bacterium decreased gradually. The final contents of DNA, RNA and protein nitrogen per bacterium were one third, one tenth and one third, respectively, of those found in cells in the logarithmic stage. In this final stage, cells assumed a smaller spherical form (Fig. 4). The ratio of the RNA to DNA declined to 2 : 1 in this stage as previously observed by McFall *et al.* (*18*).

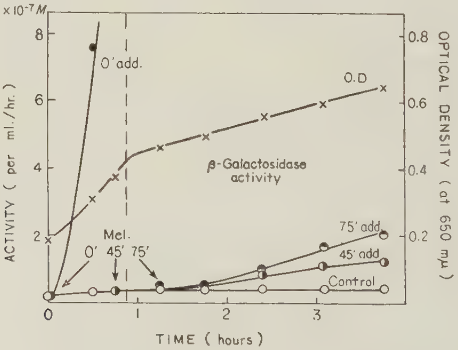

FIG. 5. β-Galactosidase formation during the phosphate deficient phase.

The seed culture was inoculated in a peptone medium and incubated at 37°. At various times, melibiose (final concentration: $1 \times 10^{-3} M$) was added to the separate culture. Enzyme activity was expressed as $10^{-7} M$ of *o*-nitrophenyl-β-D-galactoside hydrolysed per hour per ml. of the toluene-lysed cell suspension. The broken line indicates the time when the phosphate in the medium should be exhausted.

Enzyme Formation during the Phosphate Deficient Phase—As noticed in Fig. 3, the increment of protein nitrogen was observed although the RNA degraded during the phosphate deficient phase. It seems of interest to know whether the cells in this phase have an ability to elaborate new enzyme or not.

As illustrated in Fig. 5, when melibiose ($1 \times 10^{-3} M$) was added to the culture at the beginning of the the phosphate deficient phase (arrows 45′ and 75′ added in Fig. 5), several fold activity of β-galactosidase was induced comparing with that of the culture without melibiose. But the increment of the β-galactosidase activity per unit protein increment was less than one tenth that of the culture added with melibiose at zero time. Since several reports suggested that the inducible β-galactosidase is formed cocomitantly with the synthesis of RNA, it appears highly probable that a new RNA synthesis occurred during this stage, allowing simultaneously a remarkable degradation of RNA to take place.

Activities of some enzymes presumed to be related to the degradation of RNA *e.g.* RNase, PDase and PMase were examined. Results obtained by several experiments showed that the phosphate deficient bacteria has specific activities of 2–8 fold RNase, 2–3 fold PDase and about 100 fold PMase comparing with those of growing cells.

Since the RNase activity obtained are containing unspecific PDase activity and an error of the estimation is large for low activity sample, a precise estimation of RNase activity is difficult in this case. Because of the phosphate deficient bacteria has a high activity of PMase, it might be possible to overestimate the PDase activity when we use bis-p-nitrophenyl phosphate as substrate.

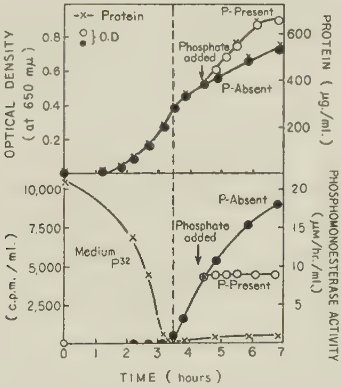

FIG. 6. Relation between phosphomonoesterase formation and concentration of phosphate in the cultural medium.

Experimental conditions were the same as described in Experimentals, except that the seed culture inoculated was one 200th. At the time indicated by the arrow the culture was divided in two portions and one 300th of original volume 1 *M* phosphate buffer (pH 7.4) was added to one culture (—○—), and the same volume of water was added to the other (—●—), and the cultures were incubated. Activity of PMase was expressed as μM of *p*-nitrophenyl phosphate hydrolysed per hour per ml. of the toluene-lysed cell suspension. Protein was estimated by the method of Lowry *et al.* (*10*). Broken line indicates the time when the phosphate in the medium was exhausted.

Although the increment of RNase and PDase activities of the phosphate deficient bacteria were not noticed, increment of the specific activity of PMase was remarkable. We investigated the relation between the formation of PMase and phosphate content in the medium.

As illustrated in Fig. 6, cells begin to form the PMase at the time when the phosphate in the medium is exhausted. Thereafter, a linear increase of PMase activity was observed for 2–3 hours and the amount of cell protein per ml. increased linearly although at a low rate. The formation of PMase was halted immediately by the addition of phoshate to the medium (final concentration; $5\times10^{-3}M$ phosphate), though the amount of protein increased at a high rate. The appearance of PMase activity was not observed in the phosphate rich; peptone-phosphate medium. It is to be noticed that the phosphate inhibit the action itself of the PMase. Complete inhibition of the PMase formation by the addition of chloramphenicol ($1\times10^{-4}M$) was also observed.

Stability of Bacterial Cells in a Phosphate Deficient Phase—Whenever the cells in the phosphate deficient phase, are resuspended in the phosphate rich medium, no lag of growth is observed, and a large portion of phosphate deficient cells remain unlysed when visualized under a microscope. These results will indicate that the RNA degradation in the phosphate deficient phase do not depend upon the lysis of the cells.

In order to confirm the above indication, an attempt has been made to determine whether or not there is enough lysis in the population of phosphate deficient bacteria to account for the 20–30 per cent degradation of RNA. Cells containing considerable quantities of melibiose-induced β-galactosidase were washed twice to remove melibiose and suspended in the peptone medium.

TABLE I

Conservation of Intracellular β-Galactosidase Activity in the Phosphate Deficient Phase

Time (*hours*)	Intracellular β-galactosidase activity (*m*μM)	Extracellular β-galactosidase activity (*m*μM)	Constitutive β-galactosidase activity (mμM)
0	9,200	25	500
1.5	9,600	50	750
3.0	9,200	55	1,000
4.5	9,200	60	1,200

Cells containing a considerable amount of melibiose-induced β-galactosidase were washed to remove melibiose and suspended in a peptone medium and incubated for several hours at 37° with aeration by shaking. In the case of detection of constitutive β-galactosidase, cells not induced were used. Enzyme activity is expressed as mμM of *o*-nitrophenyl-β-D-galactoside hydrolysed per hour per ml. of the toluene-lysed cell suspension. Extracellular enzyme activities were assayed for activities of the supernatant fraction obtained by centrifugation ($5,000\times g$ for 5 minutes). The zero time indicates the time when the phosphate in the medium was exhausted.

As shown in Table I, intracellular β-galactosidase was conserved without any appreciable loss of activity during the phosphate deficient phase, and the increment amount of extracellular β-galactosidase was negligible (less than 0.1 per cent per hour). But this bacterial strain (control) produces a small amount of constitutive β-galactosidase (the third column in the Table). If Constitutive β-galactosidase was produced similarly to control under this condition, the amount of intracellular β-galactosidase would increase to some extent. But the increment of the intracellular β-galactosidase was not observed (the first column of the Table). In case this fact was depending on the cell lysis, the lysed cells should not be more than 2 per cent per hour, showing that such a degree of degradation of RNA (20–30 per cent per 3 hours) can not be explained by the lysis of bacterial cells.

DISCUSSION

The phosphate economy in this experiment will be considered at first. At the phosphate exhaustion time, 65 per cent of the total phosphate incorporated into RNA fraction and then about 25 per cent of them (about 15 per cent of the total) degraded during the phosphate deficient phase. During this phase, about 60 per cent of the phosphate released from RNA fraction must be incorporated into the DNA fraction, and the remaining 30 per cent was splitted into the acid soluble and the medium fraction. It seems likely that a large portion of the P^{32} released from RNA is utilized for the synthesis of DNA in *E. coli* as suggested by Caldwell and Hinshelwood (*19*) using *Aerobacter aerogenes.*

The patterns of RNA-P^{32} and of DNA-P^{32} were very similar to those obtained by orcinol and by Dische's, respectively, and by the absorption at 260 mμ. Therefore, the probable contamination of lipid-P and of polyphosphate-P did not interfere with the present explanation and also the possibility that Bactopeptone in the medium contained utilizable phosphorus can be neglected. Yoshida and Sevag (*20*) have recently fractionated *E. coli* B. grown in a synthetic medium and proposed that RNA can be utilized as phosphate source in a phosphate deficient phase.

The process of the transfer of RNA-P^{32} to DNA-P^{32} can be considered in several ways. Astrachan and Volkin (*21*) suggested that T2 phage infected *E. coli* might convert RNA into DNA through ribonucleotides (or polymerized ribonucleotides). The same explanation may be tenable in the present experiment. On the other hand, in the phosphate deficient phase as shown in this experiment, such a plenty of PMase was formed that the phoshate released may have been immediately incorporated into DNA.

It was already discussed that the RNA degradation did not depend upon the lysis of the cells. Despite the excellence of the method to determine a minute cellular lysis by the decrease of preformed intracellular β-galactosidase (*22*, *23*), a great amount of β-galactosidase can be fixed to the cell debris and precipitated by centrifugation in the case of lysis using toluene or xylene (*24*). If it occurred in the phosphate deficient phase, it would be dangerous to take the data as such. However, it is not known whether and if any in what mode a lysis takes place in the phosphate deficient phase, and therefore, the above method was adopted for the time being.

It was reported that a decrease of 40*s* particles was observed in *E. coli* under the amino acid starvation (*25*) or by incubation in phosphate buffer following X-ray irradiation (*26*). In the phosphate deficient bacteria, too, a decrease of larger particles 40*s* and 29*s*) in the ultracentrifugal and of large nucleoprotein fraction in the electrophoretical pattern were observed. Moreover, it was reported that the turnover of cellular components would be appreciable in the case of defficience of some essential components (*1*, *22*, *27*, *28*). It will be interesting to compare the decrease of larger particle in this experiment with this appreciable turnover in the resting cells.

The RNA synthesized in the presence of chloramphenicol was conserved by the presence of chloramphenicol (*29–31*). In the phosphate deficient bacteria, too, the RNA was conserved by the presence of chloramphenicol. However, it still remains obscure whether the chloramphenicol conserves really the RNA itself or it synthesizes a new RNA using the material released from the preformed RNA.

It is of interest that the amount of protein per ml. increases to about 2 fold during the phosphate deficient phase where the degradation of RNA was observed (Fig. 3). Recently several facts were accumulated that a marked amount of protein synthesis is observed under the condition showing a negligible increase of RNA (*2*, *20*, *28*, *32*, *33*). Precise conditions in these experiments were different from one another and therefore, a unitary explanation is impossible. But at least in the present experiment a suggestion was given that a new RNA synthesis equivalent to the increment of protein is not necessary. Nevertheless, a new RNA synthesis must have occurred, though in a small amount, since β-galactosidase was formed in the presence of melibiose even in this phosphate deficient phase. β-galactosidase formation does not tell directly the concomitant synthesis of RNA. But many suggestions (*34–37*) have been accumulated showing a concomitant RNA synthesis with β-galactosidase induction. Therefore, an undetectable amount of RNA must have been synthesized. A similar case may be considered for the PMase formation.

It is to be noticed that PMase formation was stimulated by the exhaustion of phosphate and stopped by the addition of phosphate and that the phosphate inhibits the action itself of the enzyme. This phenomenon can be considered in relation to "the negative feed back mechanism" or the phenomenon of "the enzyme repression" (*e.g. 38–42*). Detailed studies in this connection are now under way.

SUMMARY

1. In the deficient poor medium, RNA of *E. coli* degraded by 20-30 per cent during 3-4 hours following the phosphate exhaustion in the medium. Concomitantly, the amount of the bacterial DNA and protein doubled.

2. The amount of macromolecular components containing the major part of RNA in phosphate deficient cells were diminished comparing with that in growing cells.

3. Chlorampenicol ($1 \times 10^{-4} M$) conserved the RNA and arsenate ($5 \times 10^{-3} M$) stimulated the degradation. The effect of dinitrophenol ($2 \times 10^{-3} M$) was variable. NaN_3 ($5 \times 10^{-3} M$) did not show any considerable effect.

4. During this phosphate deficient period an appreciable amount of β-galactosidase was induced by melibiose and a marked increment of phosphomonoesterase was observed.

5. The phosphomonoesterase began to be formed at the time of phosphate exhaustion in the medium and was stopped by the addition of phosphate or chloramphenicol ($1 \times 10^{-4} M$).

6. In view of the above results obtained some consideration about the phosphate economy in the cells, the relation of the RNA and protein synthesis and the negative feed back mechanism of phosphomonoesterase, were discussed.

The author is deeply indebted to Dr. D. Mizuno, the Chief of Department of Biochemistry, for his valuable discussion and encouragement, to Dr. J. Tomizawa, for his helpful criticism and advice to Mrs. S. Horiuchi, for her competent technical assistance and also during the course of this investigation.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

A PROTEOLYTIC ENZYME OF STREPTOMYCES GRISEUS

III. HOMOGENEITY OF THE PURIFIED ENZYME PREPARATION*

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(Received for publication, April 13, 1959)

In the preceding papers (*1*, *2*), convenient methods for isolating the protease of *Streptomyces griseus* from the usual culture broth were reported. Through these methods, a highly purified enzyme preparation was obtained.

Homogeneity of the preparation was then examined by ultracentrifugal, electrophoretic and enzymological analyses. The experimental results obtained have led to the conclusion that the preparation is a homogeneous, as described in the present paper.

MATERIALS AND METHODS

Enzyme—Purification of *St. griseus* protease was performed mainly as previously reported (*2*). The outline of purification procedures is shown in Scheme 1.

Ultracentrifugal Analysis—Using a Spinco-type ultracentrifuge (model-E), the sedimentation diagram of the protease was observed at 59,780 r.p.m. at 20°. The protease solution used for the analysis was prepared as follows; 20 mg. of the highly purified preparation (Preparation No. VI) was dissolved in 2 ml. of 0.1 *M* sodium acetate buffer (pH 7.0, containing 0.02 *M* calcium acetate) and dialyzed against 1 liter of the same buffer solution at 4° overnight.

Electrophoretic Analysis—Experiments were carried out at pH values of 5–9 in a Tiselius apparatus (Hitachi-type, model-HTD) at 7°. About 2 ml. of solutions containing 3 per cent of the protease (Preparation No. VI) were made up in appropriate buffers and dialyzed in cellophane tubes for 24 hours at 4° against 2 liters of the corresponding buffer solution. The outside buffer solutions were used to fill the upper compartments of the Tiselius apparatus.

Assay for Casein Digestion Activity (*Proteinase Activity*)—The enzyme activity for digesting casein was determined by the casein-Folin color method described fully in the preceding paper (*1*). The enzyme activity assayed by this method represents the apparent "proteinase activity" and is indicated by *PU* values as previously described (*1*).

Assay for Peptide Bond Hydrolysing Activity (*Peptidase Activity*)—Casein-formol titration method was adopted for this purpose. Five ml. of the enzyme solution suitably diluted with 0.02 *M* calcium acetate solution and containing about 1.5×10^{-2} *PU* was added to 5

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* Most of this work were reported at the Regular Meeting of the Agricultural Chemical Society of Japan at the University of Tokyo in November, 1958 and a part of this communication was published in Japanese in the *Scientific Papers of the Institute of Physical and Chemical Research*, **34**, 399 (1958)

SCHEME 1

Purification of St. griseus Protease

Number of preparation	Preparation	Purification process
	Culture broth	
		Exclusion of mycelia by filtration with filter-press
I	Culture broth filtrate	
		Adsorptive exclusion of streptomycin and coloring matter by resin column treatment with Kaken C-1(SP-7) resin
II	Effluent from Kaken C-1(SP-7) resin column	
		Adsorption and elution of protease by resin column treatment with Kaken C-1(SP-3) resin
III	Eluate from Kaken C-1 (SP-3) resin column (High-activity fraction)	
		Salting out with ammonium sulfate (40 g./dl.), Dialysis against dil. Ca-acetate solution
IV	Solution of $(NH_4)_2SO_4$ precipitate	
		Decolorization witn active carbon, Fractional precipitation with acetone (50-66% of acetone concentration), Dialysis against Ca-acetate solution
V	Solution of acetone precipitate	
		Lyophilization
VI	Lyophylized preparation (Highly purified preparation)	

ml. of 3 per cent casein solution (pH 7.4, containing $M/15$ sodium phosphate buffer) and kept at 30° for 20 minutes. After reaction, 10 ml. of 35 per cent formol solution (pH 6.8) was added to the mixture and electrometric titration was carried out with 0.02 N sodium hydroxide. A pH 8.5 was adopted as the end point of the titration and the titration values were corrected for the values of blanks which were measured by mixing the substrate solution with the formol solution followed by addition of the enzyme.

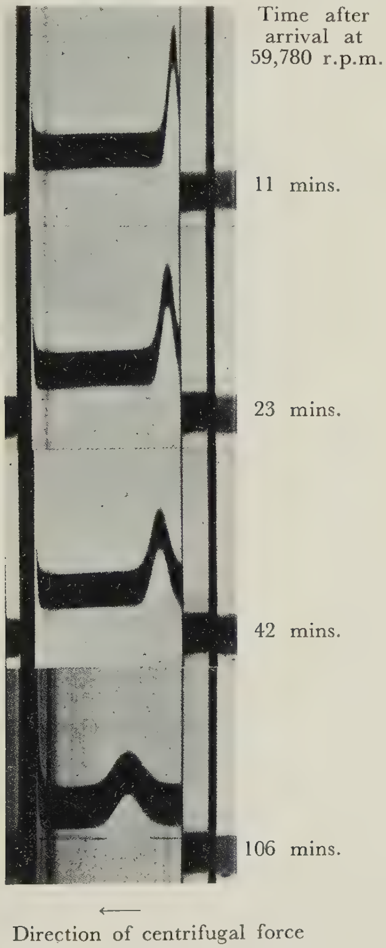
One unit of the peptidase activity was defined as that amount of enzyme which produces 1 micro-equivalent of free carboxylic acid in 1 minute's reaction under the above assay condition and represented as $FU\,{}^{\text{cas., }30^\circ\text{, formol}}_{\mu\text{. eq. COOH}}$ (Abbreviation : *FU*). The *FU*, therefore, can be calculated from the titration values as follows:

$$FU=\frac{\text{ml. of } 0.02\,N \text{ NaOH (titration value)}\times 5}{20 \text{ (reaction time)}}=\text{titration value }\times\frac{1}{4}.$$

Measurement of the Hydrolysis Extent—Five ml. of the enzyme solution (containing about 3×10^{-3} *PU* per ml.) was added to 5 ml. of 3 per cent casein solution (pH 7.4) and kept at 40° for 66 hours. After reaction, 10 ml. of 35 per cent formol solution was added to the mixture and titrated electrometrically with 0.02 N sodium hydroxide as described before. The titration values were corrected for the blanks and represented as microequivalent of free carboxylic acid which was liberated from the substrate throughout the hydrolysis.

RESULTS AND DISCUSSION

Ultracentrifuge Studies—On account of the relatively low molecular weight of this enzyme, long time intervals were needed to obtain a sufficient displacement of the boundary. During that time a considerable amount of diffusion took place, making it difficult to obtain a sharp concentration gradient on the boundary. As shown in Fig. 1, however, only one moving component was clearly observed, and the symmetry of the curves indicated the fair homogeneity of the material.



Solvent; 0.1 *M* sodium acetate buffer, containing 0.02 *M* calcium acatate, pH 7.0. Enzyme concentration; 1 per cent. r.p.m.; 59,780 r.p.m. in Spinco-type ultracentrifuge. Temperature; 20°. Diagonal bar angle; 60°.

FIG. 1. Ultracentrifugal diagram of *St. griseus* protease (Preparation No. VI).

The sedimentation constant ($s_{20, w}$) of the protease was calculated from the diagram in Fig. 1 and was determined as 2.8 S: The S is the Svedberg constant and has the dimension of 10^{-13} (cm./sec.)/(dyne/g.).

Electrophoresis Studies—As shown in Fig. 2, the electrophoretic patterns of the preparation in the Tiselius apparatus indicated the presence of only one moving component at pH 7.15 in 0.1 *M* sodium acetate buffer. A similar result was obtained at pH 8.0 in 0.1 *M* veronal buffer.

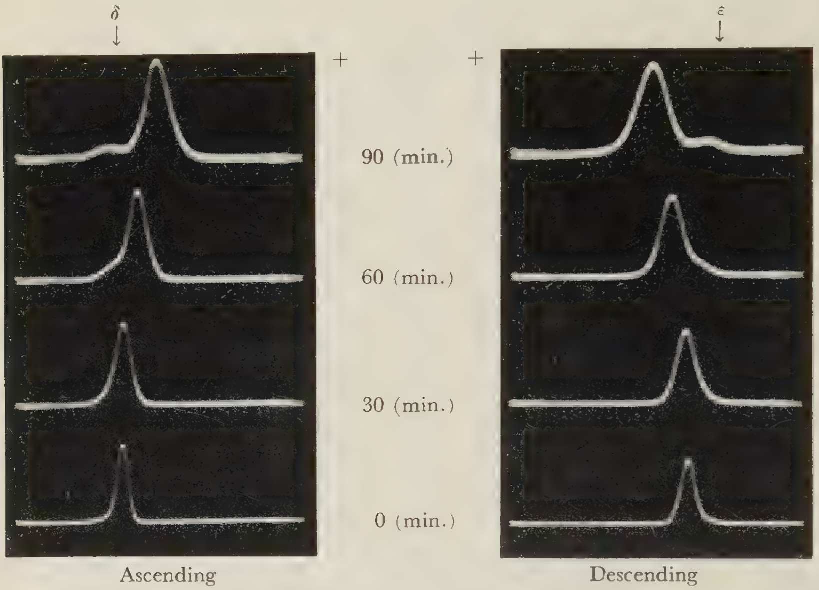


FIG. 2. Electrophoretic patterns of *St. griseus* protease (Preparation No. VI). Solvent; 0.1 *M* sodium acetate buffer, containing 0.02 *M* calcium acetate, pH 7.15, ionic strength (μ) 0.16. Protein concentration; 3 per cent. Dialysis; 24 hours at 4°. Electrophoresis; at 5 mA., 114 v., 7° in Tiselius apparatus.

The δ and ε boundaries are concentration gradients remaining near the initial boundary positions and do not represent electrically inert material.

At pH 5.6 or 8.5, however another small peak was observed. Although it was not definitely corroborated, there is a strong probability that this secondary peak was due to the modfication of the enzyme itself during the dialysis or the electrophoresis for a long time under the rather unfavorable pH for the enzyme, because similar results were obtained even at the neutral pH when the time intervals for the dialysis were prolonged more than 24 hours. Therefore, there is every indication that the material may be considered to be a homogeneous substance as an electrolyte.

Enzymological Studies (*Problem of the Contamination of Peptidases in the Preparation*) –As will be reported fully in the subsequent papers, *St. griseus* protease was found to have a very broad specificity and is able to cleave almost all kinds of peptide-linkage in proten. Since such a broad specificity has not yet been known among the other reported proteases, it was suspected that some peptidases might still be contaminating the highly purified preparation of *St. griseus* protease. In order to ascertain whether or not this was the case, the following experiments were conducted.

The first experiment adopted for this purpose was to determine the proteinase activity (*PU*) and the peptidase activity (*FU*) of the protease preparations respectively, which were collected during the process of purification, and to compare the *PU/FU* values of the preparations with one another throughout the course of purification. If there are any petidases originally contained in the culture broth together with the protease, the *PU/FU* values should be changeable and should show a tendency to increase with the progress of purification for the protease.

As shown in Table I, the experimental results indicate that the *PU/ FU* values of the preparations are almost invariable throughout the course of purification. This information suggests that not only the highly purified preparation but also the crude preparation such as culture broth itself is composed of only one kind of proteolytic enzyme which has a very broad specificity.

TABLE I

Comparison of the PU/FU Values of the Preparations of St. griseus Protease

Number of preparation[1]	Preparation[1]	Purity of preparation (*PU/PN*)	Proteinase activity[2] (*PU/ml*)	Peptidase activity[3] (*FU/ml.*)	*PU/FU* value	%
			$\times 10^{-3}$		$\times 10^{-3}$	
I	Culture broth filtrate	1.2	8.74	6.18	1.42	100
II	Effluent from Kaken C-1(SP-7) resin column	1.6	7.95	5.22	1.52	107
III	Eluate from Kaken C-1(SP-3) resin column	14.1	45.92	32.75	1.40	99
IV	Solution of $(NH_4)_2SO_4$ precipitate	16.8	94.44	61.20	1.54	108
V	Solution of acetone precipitate	26.5	276.53	189.80	1.46	100
VI	Solution of lyophilized proparation	25.2	518.50	362.05	1.43	100

1) See Scheme 1.

2) Productivity of TCA-soluble digestion product from casein per minute's reaction, assayed by the casein-Folin color method.

3) Productivity of free COOH from casein per minute's reaction, assayed by the casein-formol titration method.

The second experiment was attempted from a different standpoint. In general, the extent of enzymatic hydrolysis of a given protein depends upon the nature of protease to be used because of its specificity. When a protease preparation was contaminated with the other kind of protease (or peptidase), a marked increase in hydrolysis will be resulted. Conversely, if a protease

TABLE II

Comparison of the Hydrolysis Extent of Casein by the Preparations of St. griseus Protease

Number of preparation[1]	Preparation[1]	Purity of preparation (*PU/PN*)	Amount of protease used for hydrolysis (*PU/100 mg. casein*)	Extent of hydrolysis[2] (μ. *eq. COOH*)	%
I	Culture broth filtrate	1.2	$\times 10^{-3}$ 9.35	580.8	100
II	Effluent from Kaken C-1(SP-7) resin column	1.6	9.71	562.0	96
III	Eluate from Kaken C-1 (SP-3) resin column	14.1	8.67	578.4	99
IV	Solution of $(NH_4)_2SO_4$ precipitate	16.8	9.87	554.8	96
V	Solution of acetone precipitate	26.5	9.22	561.2	97
VI	Solution of lyophilized preparation	25.2	10.87	544.4	94

1) See Scheme 1.
2) Productivity of free COOH from casein in 66 hours' hydrolysis, assayed by the casein-formol titration method.

preparation is enzymologically homogeneous, the extent of hydrolysis by the preparation is expected to be constant. In other words, if the extents of hydrolysis by a number of preparations of a protease give a definite level, the preparation in question is supposed to be enzymatically homogenous.

On this assumption, the hydrolysis extents for casein by the preparations of *St. griseus* protease collected during the purification process were compared with one another (Table II). As shown in Table II, it was confirmed that the hydrolysis extents by the preparations agreed with one another fairly well throughout the course of purification. This finding also supports the above mentioned consideration that the proteolytic enzyme system of *St. griseus* is naturally composed of only one kind of protease (*i.e.*, proteinase).

SUMMARY

St. griseus protease was obtained as a highly purified preparation by the previously reported methods. The homogeneity of this preparation was confirmed as follows:

1. The preparation was found to be composed of only one moving component by ultracentrifugal analysis.

2. The electrophoresis indicated that the preparation is negatively charged and moves as a single electrophoretic component at neutral pH. Although another small peak appears at rather unfavorable pH for the enzyme, this abnormality may be caused by the modification of the enzyme itself during the dialysis.

3. The preparation was also proved to be enzymologically homogeneous and not contaminated by any other kind of peptidase or proteinase. Naturally, the proteolytic enzyme system of *St. griseus* seems to be composed of the single protease which has a very broad specificity.

The authors wish to express their thanks to Dr. T. Akahira, Dr. M. Yanagita, Prof. K. Sakaguchi, Prof, K. Okunuki and Dr. B. Hagihara for their kind guidances and useful suggestions. The authors are also grateful to Mr. S. Fujita for preparation of enzyme, to Mr. H. Tochihara for ultracentrifuge analyses and to Mr. M. Murakami for his helpful assistance.

The present work was aided in part by the grant of Scientific Research Fund of the Ministry of Education for which the authors wish to thank.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

STUDIES ON ACTIVE GROUPS OF PAPAIN

I. EFFECT OF ACTIVATORS ON INHIBITION BY ALDEHYDE REAGENTS*

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(Received for publication, April 13, 1959)

Since Vines (*1*) demonstrated the activation of papain by hydrogen cyanide, various activators of papain have been investigated and the importance of the thiol group in the enzyme has been confirmed by many workers (*2–13*). Recently Smith (*14*) proposed an improved hypothesis for the reaction mechanism for the hydrolytic action of papain. This was developed from a tentative mechanism for the hydrolysis of benzoyl-L-arginine-amide (*15*). By this mechanism, an intramolecular thiol ester is considered as the active binding site with the carbonyl carbon of the peptide bond.

Bergmann and Ross (*16*) found that phenylhydrazine inhibits the hydrolysis of synthetic substrates but does not inhibit gelatin-digestion by papain. They therefore proposed that there are two types of enzyme in the papain-complex, the one having a proteolytic action and the other a peptidase action. The latter action involves an aldehyde group as the active site.

Okumura (*17–19*) reported that the activity of papain is progressively inhibited with time on treatment with aldehyde reagents, such as hydroxylamine, phenylhydrazine, sodium bisulfite, or *NN'*-dimethylbarbituric acid, finally losing the total activity. He concluded that there is only one enzyme in the papain-complex, with an aldehyde group as the active group. The existence of the aldehyde group was supported chiefly by the reaction of papain with *NN'*-dimethylbarbituric acid.

However, Durell and Fruton (*20*) found that hydroxamic acid is formed by transpeptidation by papain in the presence of hydroxylamine and certain synthetic substrates. In this case, there was no inhibition by hydroxylamine. These two results seem to be in disagreement. However there was some difference in experimental conditions between the above two investigations, such as the substrates and activating reagents used. Particularly it seems very likely that the activating reagents cause a difference in susceptibility of papain to aldehyde reagents.

To clarify the interrelation of activators with the susceptibility to aldehyde reagents in the present investigation a study was made of the effect of aldehyde reagents on several forms of papain activated with various agents.

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* An outline of this work was given at the 9th Meeting of Symposia on the Structure of Proteins in Osaka, in November, 1958.

EXPERIMENTALS

Crystalline Papain—Crystalline papain was prepared from dried papaya latex, essentially by the method of Kimmel and Smith (*12*). Deionized water was used instead of cysteine solution throughout. The preparation was twice recrystallized before use.

Mercuripapain –Mercuripapain was prepared from twice recrystallized papain according to the method of Kimmel and Smith (*12*).

Determination of Mercury—Mercury was determined by the method of Sandell (*21*), using di-β-naphthylthiocarbazone.

Assay for Proteolytic Activity of Papain—The proteolytic activity of papain was determined by the following method, essentially based on the methods of Anson (*22*) and Kunitz (*23*). One millilitre of enzyme solution, containing suitable amounts of enzyme, buffer, activators, and/or inhibitors, was mixed with 1 ml. of 1.0 per cent casein solution. After 10 minute incubation at 30°, 2 ml. of 0.44 M trichloroacetic acid was added to the reaction mixture and after standing at 30° for about 20 minutes, the resulting suspension was filtered. The optical density of the filtrate at 275 mμ was measured by a Beckmann type spectrophotometer, using a 1 cm. cuvette. The readings were corrected for the value of blanks, in which the enzyme had been mixed with 0.44 M trichloroacetic acid before the addition of substrate. The enzyme activity is expressed as this corrected reading of the optical density of the filtrate at 275 mμ.

RESULTS

As reported by Okumura (*17–19*), untreated or cyanide-activated papain is apparently inhibited by aldehyde reagents, such as phenylhydrazine, hydroxylamine, sodium bisulfite, and semicarbazide (Table I). The inhibition

Table I

Effect of Aldehyde Reagents on Cyanide-activated Papain

Enzyme concentration; 0.0078 per cent. 0.1 M phosphate buffer, pH 6.1. Total volume; 2.0 ml. In Exp. 1, enzyme was incubated with potassium cyanide (adjusted to pH 6.1) at 30° for one hour and then incubated with each aldehyde reagent. After 30 minute incubation activity was assayed. In Exp. 2, enzyme was incubated with aldehyde reagents at 30° for 30 minutes, and then added with potassium cyanide. After one hour incubation, activity was assayed. Potassium cyanide was at 5×10^{-2} M final concentration.

Exp. No.	Reagent added	Activity
1	——	.860
	2.5×10^{-3} M phenylhydrazine	.394
	,, semicarbazide	.736
	10^{-2} M sodium bisulfite	.719
	5×10^{-3} M hydroxylamine	.296
2	2.5×10^{-3} M phenylhydrazine	.091
	,, semicarbazide	.651[1]
	10^{-2} M sodium bisulfite	.241
	5×10^{-3} M hydroxylamine	.033

1) Though semicarbazide is a feeble inhibitor, activity was almost completely lost, after 24 hour incubation.

was progressive with time and after 24 hour incubation with semicarbazide which was the most feeble inhibitor, the activity of papain was almost com-

TABLE II

Effect of Aldehyde Reagents on Cysteine-activated Papain

Enzyme concentration; 0.0055 per cent. 0.1 M phosphate buffer, pH 6.1. Total volume; 2.0 ml. In Exp. 1, enzyme was incubated with aldehyde reagents and cysteine at 30° for 30 minutes and then activity was assayed. In Exp. 2, enzyme was incubated with aldehyde reagents at 30° for 30 minutes and then added with cysteine. After 10 minute incubation, activity was assayed. Cysteine was at 2.5×10^{-3} M final concentration.

Exp. No.	Reagent added	Activity
1	——	.223
	5×10^{-3} M hydroxylamine	.221
	2.5×10^{-3} M phenylhydrazine	.216
	,, semicarbazide	.212
	10^{-2} M sodium bisulfite	.223
2	5×10^{-3} M hydroxylamine	.111
	2.5×10^{-3} M phenylhydrazine	.093
	,, semicarbazide	.093
	10^{-2} M sodium bisulfite	.053

TABLE III

Effect of Aldehyde Reagents on Thioglycolate-activated Papain

Enzyme concentration; 0.019 per cent in Exp. 1 and 0.006 per cent in Exp. 2. 0.1 M phosphate buffer, pH 6.1. Total volume; 2.0 ml. In Exp. 1, enzyme was incubated with thioglycolate (adjusted to pH 6.1) at 30° for 5 minutes and then aldehyde reagents were added. After 20 minutes incubation, activity was assayed. In Exp. 2, enzyme was incubated with aldehyde reagents at 30° for 30 minutes and then added with thioglycolate. After 5 minutes incubation, activity was assayed. Thioglycolate was at 5×10^{-5} M final concentration.

Exp. No.	Reagent added	Activity
1	——	1.264
	2.5×10^{-3} M hydroxylamine	1.207
	,, phenylhydrazine	1.280
	,, semicarbazide	1.286
	5×10^{-3} M sodium bisulfite	1.090
2	——	.578
	2.5×10^{-3} M hydroxylamine	.318
	,, phenylhydrazine	.272
	,, semicarbazide	.521
	5×10^{-3} M sodium bisulfite	.081

pletely lost under the present conditions. When the enzyme was treated with aldehyde reagents prior to cyanide-activation, it was more strongly inhibited (Table I).

Since Kimmel and Smith (*12*) reported that cysteine is a better activator than cyanide, the effect of aldehyde reagents on cysteine-activated papain was investigated. As shown in Table II, cysteine-activated papain was hardly inhibited by aldehyde reagents. From these results, it is conceivable that the activation of papain by reducing agents involving a sulfhydryl group differs from that by cyanide. If this is the case, it is reasonable to assume that thioglycolate and hydrogen sulfide will behave like cysteine towards aldehyde reagents, because they also were known to activate papain (*6*, *7*). The effect of aldehyde reagents on thioglycolate- and hydrogen sulfide-activated papains was therefore investigated.

As can be seen in Table III, thioglycolate-activated papain also was hardly affected by aldehyde reagents. Moreover, as with the case of cysteine- and thioglycolate-activated enzyme, hydrogen sulfide-activated papain was not inhibited by aldehyde reagents (Table IV). Addition of potassium bisulfite caused great activation rather than inhibition. Under the present conditions, papain was not completely activated by hydrogen sulfide. The above activation might be caused by some reducer formed during the reaction of bisulfite with hydrogen sulfide.

TABLE IV

Effect of Aldehyde Reagents on Hydrogen Sulfide-activated Papain

Enzyme concentration ; 0.0404 per cent. 0.1 *M* phosphate buffer, pH 6.1. Total volume ; 2.0 ml. Enzyme was activated with hydrogen sulfide and then added with aldehyde reagents. After one hour incubation at 30°, activity was assayed. Activation time ; 20 minutes in Exp. 1 and 5 minutes in Exp. 2.

Exp. No.	Reagent added	Activity
1	——	.485
	2.5×10^{-3} *M* hydroxylamine	.407
	,, phenylhydrazine	.395
	,, semicarbazide	.427
	5×10^{-3} *M* sodium bisulfite	1.133
2	——	.495
	2.5×10^{-3} *M* hydroxylamine	.478
	,, phenylhydrazine	.468
	,, semicarbazide	.482
	5×10^{-3} *M* sodium bisulfite	1.331

Cysteine was used as activator of papain in the experiment of Durell and Fruton, while Okumura used untreated papain and cyanide-activated enzyme in his experiments. Therefore the results may resolve the contradiction between the reports of Okumura and of Durell and Fruton.

Furthermore, it was noted that agents such as cysteine and thioglycolate could not reactivate enzyme already inhibited by aldehyde reagents.

Since mercuripapain has been reported to bind on its active sulfhydryl group (*12*, *13*), the effect of aldehyde reagents on mercuripapain was investigated. The mercurienzyme was apparently inhibited by these reagents (Table V).

TABLE V

Effect of Aldehyde Reagents on Mercuripapain

Enzyme concentration; 0.0138 per cent. 0.1 M phosphate buffer, pH 6.1. Total volume; 2.0 ml. Enzyme was incubated with aldehyde reagents at 30° for 45 minutes and then added with cysteine. After 5 minutes incubation, activity was assayed. Cysteine was at $2.5 \times 10^{-3}\,M$ final concentration.

Reagent added		Activity
	——	.842
$5 \times 10^{-3}\,M$	hydroxylamine	.476
$2.5 \times 10^{-3}\,M$	phenylhydrazine	.000
,,	semicarbazide	.856
$5 \times 10^{-3}\,M$	sodium bisulfite	.092

TABLE VI

Inhibition of Mercuripapain by Phenylhydrazine followed by No Liberation of Mercury

Reaction mixture A; 0.207 per cent mercuripapain and 0.1 M phosphate buffer (pH 6.1). Reaction mixture B; 0.207 per cent mercuripapain, 0.1 M phosphate buffer (pH 6.1) and $10^{-2}\,M$ phenylhydrazine. Total volume; 5.0 ml. 0.1 ml. aliquots were removed from the original mixture at intervals and added with 0.8 ml. of 0.1 M phosphate buffer (pH 6.1) and 0.1 ml. of $5 \times 10^{-2}\,M$ cysteine. After 5 minutes, activity was assayed. For determination of mercury, 1.0 ml. aliquots were removed from the original mixture at intervals and added with 1.0 ml. of 0.44 M trichloroacetic acid. After standing for about 20 minutes, the mixture was centrifuged and washed twice with 0.22 M trichloroacetic acid. The precipitate and the supernatant were separately ashed and the mercury content of each was determined.

Flask No.	Time in minute	Activity	Mercury in	
			supernatant (μg./ml.)	precipitate (μg./ml.)
A	5	1.213	—	—
	30	1.221	—	—
	60	1.203	0.0	9.9
B	5	.920	0.0	—
	30	.063	0.0	—
	60	.047	0.0	9.5

It was noticed that in the course of inhibition no mercury was released from the enzyme (Table VI). Mercury was, however, recovered in the enzyme protein after inhibition and precipitation by trichloroacetic acid. Therefore it is concluded that the group which reacts with aldehyde reagents differs from the sulfhydryl group binding mercury.

DISCUSSION

At present, it is recognized that a thiol group plays an important part in the proteolytic action of papain and the activation of the enzyme depends on the appearance of an active thiol group.

On the other hand, results which suggest the existence of an aldehyde group in the enzyme have been reported by Bergmann and Ross (*16*). The importance and the existence of this aldehyde group were confirmed by Okumura (*17–19*), using *NN'*-dimethylbarbituric acid which reacts specifically with aldehydes. The results obtained in the present investigation agree with those of Okumura on the existence of an aldehyde group in papain. However an aldehyde group seems not always to be present in various kinds of papain, because cysteine-, thioglycolate-, and hydrogen sulfide-activated papains were not inhibited by aldehyde reagents. It is conceivable that the aldehyde group is present only in untreated papain. No inhibition of activated enzymes other than that of cyanide-activated papain by aldehyde reagents was observed. This suggests that activation by cysteine, thioglycolate, and hydrogen sulfide causes the conversion of the aldehyde group into some form which does not react with aldehyde reagents. Aldehyde might react with cysteine or thioglycolate to form a compound like mercaptal. In cyanide-activated papain, the aldehyde group is probably converted into cyanohydrin, which is known to react with the NH_2-group of hydroxylamine, phenylhydrazine, or semicarbazide and to be substituted with bisulfite. The above assumption could explain the inhibition of cyanide-activated papain by aldehyde reagents.

A difference between activation by cyanide and that by other reducing agents such as cysteine was observed by Matsuyama and Shimura (*24*) and Irving *et al* (*25*). Matsuyama and Shimura found that the rate of gelatin-digestion by cyanide-activated papain was lower than that by cysteine-activated papain at the beginning of digestion and the former became higher than the latter at a later stage. These workers supposed that cyanide-activated papain would be active in the peptidatic action and cysteine-activated form in the proteolytic action. However, it is impossible that the activating treatment of papain should cause only a difference of specificity of the enzyme, because the difference between the activation by cyanide and by other reducing reagents was also found in the hydrolysis of benzoyl-L-arginineamide. Irving *et al.* observed that the rate of hydrolysis of benzoyl-L-arginineamide by cyanide-activated papain was not proportional to the concentration of the enzyme. An increase in concentration of enzyme resulted in a marked increase of reaction rate, though with cysteine-activated papain a nearly linear relationship was obtained.

Inhibition of mercuripapain by aldehyde reagents without liberation of mercury, suggests that the group which combines with aldehyde reagents is not identical with the sulfhydryl group binding the mercury. In other words, it suggests that in the active site of papain there are in the same area two reactive groups, an aldehyde and a sulfhydryl group. However it is doubtful whether aldehyde itself acts as an active group in papain, because the cysteine- or thioglycolate-activated enzyme was not inhibited by aldehyde reagents and had a high activity.

SUMMARY

1. To clarify the interrelation of activators with the susceptibility of papain to aldehyde reagents (*i.e.* phenylhydrazine, hydroxylamine, semicarbazide, and sodium bisulfite) a study was made of the effect of these reagents on papains which had been activated in various ways.

2. Untreated papain and the cyanide-activated form were apparently inhibited by aldehyde reagents, but the cysteine-, thioglycolate-, or hydrogen sulfide-activated papains were hardly affected.

3. Mercuripapain in which papain is thought to combine with mercury through its active thiol group was also inhibited by aldehyde reagents. There was no liberation of mercury accompanying the inhibition.

4. The difference between activation by cyanide and by cysteine or thioglycolate is discussed.

The author wishes to express his gratitude to Prof. K. Okunuki and Prof. S. Akabori for their kind support in this work.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

STUDIES ON CAROTENOGENESIS BY MICROCOCCUS PYOGENES VAR AUREUS

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(Received for publication, April 17, 1959)

When *Micrococcus pyogenes var aureus* 209 P is cultured on the media which contain gradually increased amounts of tetracycline, oxytetracycline or chlortetracycline, the coccus is observed to attain a highly resisting character against these antibiotics, showing a resistance to more than 300 μg. antibiotics/ml., in contrast to the original strain which is unable to live in a medium containing 0.3 μg. of antibiotics per ml. It was noticed that, concomitantly with the acquisition of resistance towards the antibiotics, the bacterial cells happened to become colorless (*1*), a fact which is reminiscent of the phenomenon that the cells of the coccus became colorless when they were grown in a medium containing a minute amount of diphenylamine.

Regarding the process of carotenogenesis in tomato plants, Porter and Lincoln (*2*) have proposed a scheme which involves a stepwise dehydrogenation of colorless C_{40} precursors (phytoene, phytofluene) to ζ-carotene, tetrahydrolycopene (neurosporene) and δ-carotene. The process of carotenogenesis in microbial cells has not yet been studied because of the difficulty in obtaining sufficient quantities of carotenoids.

In the present study the coccus was cultivated in semi-anaerobic conditions such as in liquid cultures with relatively small areas of surface compared to the volumes of the culture solution and in aerobic condition with agar-plates; the carotenoids produced were followed spectrophotometrically under various cultural conditions, and it was found that the mechanism of carotenogenesis in *Micrococcus pyogenes var aureus* is similar to that occurring in tomato tissues. Accumulation of an unidentified 'C_{10} compound' in the coccus was also presumed by spectrophotometrical analysis. Inhibition of the bacterial metabolism by diphenylamine was also investigated, and it was inferred that the main action of diphenylamine is to inhibit the polymerization of the 'C_{10} compound' in the process of carotenogenesis.

EXPERIMENTALS

Bacterium—Micrococcus pyogenes var aureus 209 P, a strain which has been known to be sensitive to tetracyclines, was used in all experiments.

Culture Media—Glycerol medium and acetate medium shown in Table I were used for the cultivation of the cocci. The glycerol medium was adjusted to pH 7.5 with sodium hydroxide.

TABLE I

Culture Media Used for Cultivation of Micrococcus pyogenes var aureus 209 P

Glycerol medium		Acetate medium			
Peptone	10 (*g.*)	Peptone	10 (*g.*)	$MgSO_4$	0.2 (*g.*)
Beef broth	10	Beef broth	1	Nicotinamide	1.2 (*mg.*)
NaCl	2	NaCl	2	Aneurin	34 (*μg.*)
Glycerol	20	Sodium acetate	40	K_2HPO_4	9.69 (*g.*)
		Agar	25	KH_2PO_4	1.50
Total	1 liter	Total	1 liter		
pH	7.5	pH	7.5		

Method of Culture—Semi-anaerobic cultures were carried out in several ways. 100 ml. of the media were inoculated with the cocci in 300 ml. Erlenmeyer flasks. A group was incubated for twelve hours at 37°. Another group was first incubated for twelve hours at 37° and then left standing for 5 days at room temperature. Aerobic cultivation was carried out on the culture media of acetate agar in Petri plates. Fifteen hours of incubation at 37° was usually found to be enough for the maximal production of colorful carotenoids. Diphenylamine was dissolved in 80 per cent ethanol and added to the acetate agar to a final concentration of 20 μg./ml.

Extraction of the Carotenoids—Collected cells of the ordinary culture were washed twice with 0.5 per cent sodium chloride solution. The extraction of the carotenoids was effected by the procedure shown in Scheme 1.

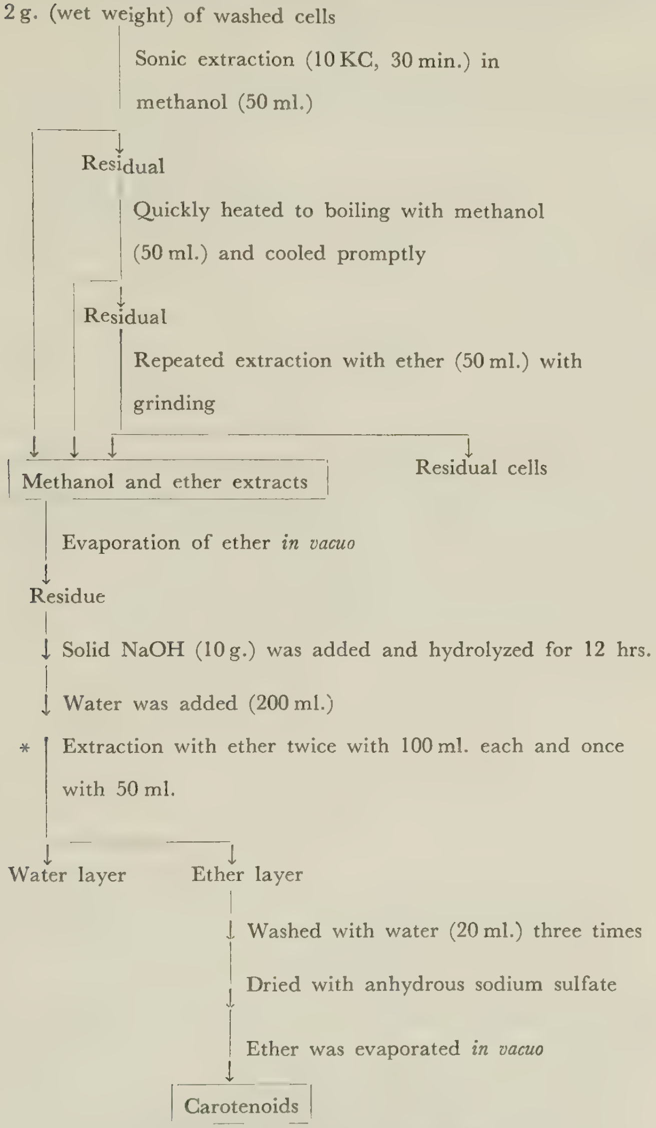
For the extraction, freshly distilled methanol was used. Ether used for the extraction was previously shaken with ferrous ammonium sulfate and freshly distilled. *n*-Hexane was washed successively with concentrated sulfuric acid and nitric acid, diluted sodium hydroxide solution, water, ferrous ammonium solution and water. The whole process of extraction of carotenoids were carried out in a dim room. Special caution was taken to prevent the effect of oxygen by replacing the air in the vessels with carbon dioxide.

Instrument—Analysis of carotenoid and measurement of dry weight of cells were conducted by using a Beckman photoelectric spectrophotometer model DU.

Column Chromatography—Calcium hydroxide (prepared by Junsei Pure Chemicals & Co., Ltd., Tokyo) and calcium carbonate (prepared by M. Nakarai Chemicals & Co., Ltd., Kyoto) of special grades were used for the column chromatography of carotenoids. The chemicals were previously heated at 250-300° for 3 hours and compactly packed in the column after cooling. The upper layer of the column consisted of calcium carbonate, and the lower part was a layer of calcium hydroxide. Each column was washed with petroleum ether (b.p. 35–70°). Petroleum ether solution of carotenoids extracted from the cells were poured into the column, and the pigments adsorbed were developed with petroleum ether and ether (9 : 1 in volume). For the elution of pigments adsorbed, the upper layer was separated from the lower one and each layer was extracted with ether. Ether was evaporated off from both eluates and the residues were again dissolved in petroleum ether. Both fractions of dissolved pigments were purified by repeated adsorption and elution with the columns of calcium carbonate or of calcium hydroxide.

SCHEME 1

Procedure of Extraction of Carotenoids from the Microbial Cells


2 g. (wet weight) of washed cells
Sonic extraction (10 KC, 30 min.) in methanol (50 ml.)
Residual
Quickly heated to boiling with methanol (50 ml.) and cooled promptly
Residual
Repeated extraction with ether (50 ml.) with grinding
Methanol and ether extracts
Residual cells
Evaporation of ether in vacuo
Residue
Solid NaOH (10 g.) was added and hydrolyzed for 12 hrs.
Water was added (200 ml.)
*
Extraction with ether twice with 100 ml. each and once with 50 ml.
Water layer
Ether layer
Washed with water (20 ml.) three times
Dried with anhydrous sodium sulfate
Ether was evaporated in vacuo
Carotenoids

* At this step the properties of the substance showing an absorption maximum at 258 mμ (in *n*-hexane) was investigated, making the solution, just before the extraction of the carotenoids with ether, neutral, basic or acidic (*cf.* Fig. 4).

RESULTS

Column Chromatographic Separation—Carotenoid pigments extracted from the cells under ordinary culture conditions were rechromatographed and identified with δ-carotene and rubixanthin (Table II) (*1*, *3*).

Absorption Spectra—The absorption spectra of carotenoids obtained by the procedure in Table II are shown in Fig. 1. Optical densities were measured at 2.5 to 0.5 mμ intervals (Fig. 1. 2. 3).

TABLE II

Absorption Maxima of the Carotenoids Separated by the Chromatography with $CaCO_3$ *and* $Ca(OH)_2$

	Absorption maxima* (mμ)		
Carotenoid adsorbed on the upper layer (Rubixanthin)	494,	462,	432.
Carotenoid adsorbed on the lower layer (δ-Carotene)	488,	456,	430.

* Absorption maxima in *n*-hexane.

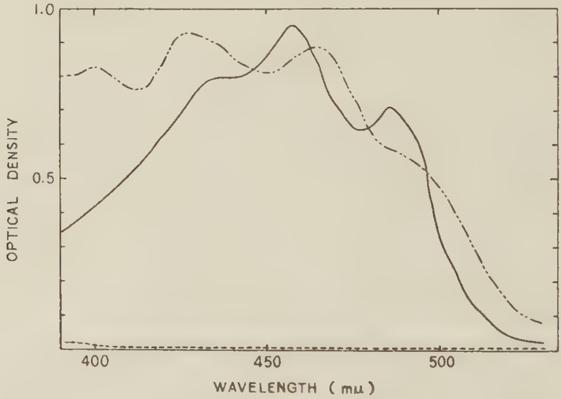


FIG. 1. Absorption spectra in the visible range of the extracted carotenoids in *n*-hexane.

The culture media, time and temperature of cultivation and concentration of the extracts were:

———; agar medium 37°, 15 hours, 20 mg. cells/ml.

----; liquid medium, 37°, 15 hours, 20 mg. cells/ml.

-—-; liquid medium, 37°, 12 hours and 5 days at room temperature, 50 mg. cells/ml.

The absorption spectra of the extracts of cells cultured at 37° or at room temperature for definite times on agar media or in liquid media by the procedure shown in Scheme 1 are presented in Fig. 1. and Fig. 2.

The absorption spectrum of the extracts of cells which were cultivated in the presence of diphenylamine (20 μg./ml.) is shown in Fig. 3.

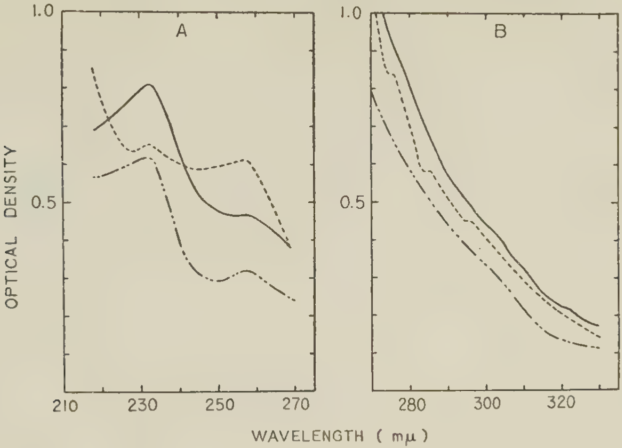


FIG. 2. Absorption spectra in the ultra-violet range of the extracted carotenoids in *n*-hexane.

Hydrolysis by sodium hydroxide was omitted in extraction procedure of Scheme 1. The culture media, time and temperature of cultivation and the final concentration of the extracts were:

——— ; agar medium, 37° 15 hours, (A) 0.1 (B) 0.3 (mg. cells/mg.),

- - - - ; liquid medium, 37° 12 hours, (A) 1.0 (B) 3.5 (mg. cell/ml.),

••—•• ; liquid medium, 37° 12 hours and 5 days at room temperature, (A) 0.3 (B) 1.0 (mg. cells/ml.).

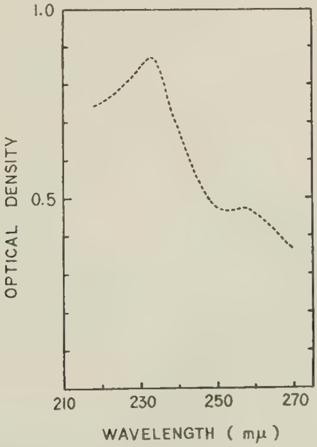


FIG. 3. Absorption spectrum of the extract of cells which cultivated by adding diphenylamine (20 μg./ml.) to the agar media. The extraction method was the same as given in Scheme 1 but hydrolysis by sodium hydroxide was omitted. Cells were cultivated at 37° for 15 hours. The concentration of the extract was 0.1 mg. cells/ml.

The cells obtained from aerobic cultivation and liquid cultivation lasting for 12 hours at 37° and 5 days at room temperature, respectively, showed a reddish-yellow color and were found to contain sizable amounts of δ-carotene and rubixanthin (Table II). Absorption maxima (in the visible range) of the carotenoids obtained from the cells grown in the liquid medium for 12 hours at 37° and 5 days at room temperature were 400, 424–429 and 465 mμ. In contrast, the cells collected after 12 hours' cultivation in the liquid medium were colorless with an absorption spectrum having no peak in the visible range but showing peaks quite identical with those of phytoene (*4*) in the ultraviolet region (Fig. 2-B). As shown in Fig. 2-A, these peaks (232.5, 258 mμ in *n*-hexane) were observed in the extracts of all samples of bacterial cells irrespective of the media and other conditions used in their cultivation.

When 20 μg./ml. of diphenylamine was previously added to the culture media, the grown cells became colorless. The extract of these cells had no peaks in the visible region except those in the ultraviolet region (Fig. 3).

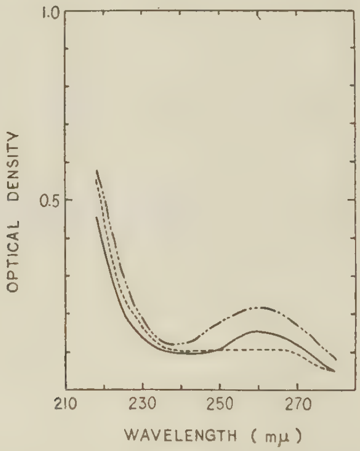


FIG. 4. Absorption spectra of the water layer obtained after repeated extraction of carotenoids with ether (*cf.* Scheme 1*). Absorption spectra were measured in water. Ten drops of the water layer fraction were added to 3 ml. of water. The final step of the carotenoids extraction with ether (*cf.* Scheme 1*) was carried out after the solution had been mad neutral (——), basic (– – – –), or acidic (••——••), respectively.

The extraction of the carotenoids with ether from the aqueous solution (*cf.* Scheme 1) was carried out after the solution had been made strongly basic. The absorption spectra of the carotenoids extracted are shown in Figs. 1 and 2. The absorption spectra of the water soluble substances given in Fig. 4 were those measured with the solutions which had been made neutral, basic or acidic, just before the extraction of the carotenoids. As shown in Fig. 4., only one absorption maximum at 260 mμ was noticed in the water soluble fraction when the solution had been made neutral or acidic. The substance showing this absorption seems, therefore, to be basic and rather lyophilic in nature. This substance is apparently different from the substance which showed an absorption maximum at 232.5 mμ in *n*-hexane (*cf.* Fig. 2-A, Fig. 3).

Judging from the characteristics of its spectrum (*5–7*), the substance which showed a high absorbancy at 232.5 mμ, may be a terpenoid compound such as geraniol, nerol, citronerol, dihydrocitronerol, citral or citronellal. Further investigations along these lines are in progress, but tentatively the substance or substances in question might be called the 'C_{10} compound' in the following discussion.

DISCUSSION

As mentioned above, phytoene is accumulated in the cells of cocci when the bacterium is grown in liquid media for a relatively short time such as 12 hours, whereas at later stages of cultivation it is replaced by ζ-carotene (absorption maxima 400, 425 mμ) (*8*) and rubixanthin. The formation of these colored substances (δ-carotene and rubixanthin) was found to occur more rapidly under aerobic conditions. These results seem to support the view proposed by Porter and Lincoln (*2*) concerning the mechanism of carotenogenesis that the formation of ζ-carotene and δ-carotene occurs by the stepwise dehydrogenation of a colorless C_{40} precursor (phytoene). Seemingly in contradiction to their views, however, tetrahydrophytoene (eicosahydrolycopene) (*2*), which has an absorption maximum at 220 mμ, could not be detected in the extracts of the cocci cells.

By comparing the absorption spectra of the ether extract of the coccus tested with those of geraniol, citral and citronellal, the presence of the 'C_{10} compound' was presumed. Among these terpenoids, geraniol and citronellol have absorption minima around 223 mμ in *n*-hexane solutions, while the substance accumulated in the cocci cells and citral do not show such absorption minima (*5*-7) (Fig. 2-A). In addition, citral shows a molar extinction coefficient (log ε 4.13) higher than those of geraniol and citronellal (*cf.* 2.29 and 1.93, respectively). These facts indicate that the substance(s) in question may most probably be related to, if not quite identical with, citral. Isolation and identification of this substance is now under investigation. The polymerization of 'C_{10} compound' to 'C_{40} carotenoids' is thought to be a process which is rate-limiting for the carotenogenesis in this coccus.

It has been proved that diphenylamine inhibits the biosynthesis of C_{40} compounds in the step or steps of polymerization of the 'C_{10} compound'.

The basic and lyophilic substance obtained from the microbial extract, which showed an absorption maximum at 258 mμ in *n*-hexane, is believed to have little relation to the process of carotenogenesis.

SUMMARY

1. The process of carotenogenesis occurring in *Micrococcus pyogenes var*

aureus 209 P was investigated spectrophotometrically. On a short-time liquid cultivation of the organism, the formation of phytoene was detected, while on its long-lasting liquid cultivation a stimulation was observed in the formation of ζ-carotene and rubixanthin. Aerobic condition was found to favor the accumulation of δ-carotene and rubixanthin even at the earlier stages of cultivation. These results suggested strongly the occurrence of a stepwise dehydrogenation in the process of carotenogenesis.

2. The coccus also proved to accumulate a substance presumably the 'C_{10} compound', showing an absorption maximum at 232.5 mμ (in *n*-hexane). Besides, it accumulated a substance of a basic character showing an absorption maximum at 258 mμ (in *n*-hexane).

3. Diphenylamine was found to inhibit the biosynthesis of C_{40} carotenoids in the step or steps of polymerization of 'C_{10} compound' in the cells of the test organism.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

ENZYMATIC SYNTHESIS OF C^{14} LABELED THYMIDINE

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(Received for publication, April 17, 1959)

It is well established by the studies of Reichard and Estborne (*1*, *2*), Friedkin and Roberts (*3*, *4*), and Kornberg *et al.* (*5*) that thymidine is a precurser of DNA but is not utilized for biosynthesis of RNA. These workers employed the thymidine labeled either with C^{14} or N^{15}. Recently, Verly *et al.* (*6*) and Taylor *et al.* (*7*) prepared the deoxynucleoside containing tritium and used for the same study. Tritium emits β ray of so low energy that the measurement of radioactivity is difficult but is excellent for microradioautographycal study. On the other hand, the radioactivity of C^{14} is easily measured by conventional G-M counter and so advantagious for tracer study.

C^{14} thymidine with high specific activity has been prepared by Friedkin *et al.* (*4*) enzymatically by the use of thymidine phosphorylase from horse liver. They prepared deoxyribose-1-phosphate from thymidine (reaction 1) and then synthesized C^{14} thymidine from C^{14}-thymine and deoxyribose-1-phosphate (reaction 2).

(1) Thymidine + inorganic phosphate $\rightleftharpoons$ thymine + deoxyribose-1-phosphate

(2) C^{14} Thymine + deoxyribose-1-phosphate $\rightleftharpoons$ C^{14} thymidine + inorganic phosphate

But their original method requires large amounts of thymidine and moreover the separation of C^{14} compounds is not easily performed under usual scale. On the relatively microscale experiment, Reichard *et al.* (*2*) devised reaction 3 as shown in the equation (*3*), the net reaction of above mentioned reaction 1 and reaction 2 with bacterial enzyme, and the C^{14} compounds are separated by ion exchange chromatography.

(3) Thymidine + C^{14} thymine + orthophosphate $\rightleftharpoons$ C^{14} thymine + C^{14} thymidine + orthophosphate

Besides above mentioned methods, Schweigart *et al.* (*8*) isolated C^{14} thymidine from DNA of bacteria which cultivated under $C^{14}O_2$ atmosphere, but the thymidine is labeled universally and low specific activity.

We also reinvestigated the preparation of C^{14} labeled thymidine, and prepared it with relatively high specific activity by the method of Reichards reaction with cattle liver enzyme, and C^{14} compounds were separated by paper and ion exchange chromatography.

MATERIALS AND EXPERIMENTS

Materials—Thymidine was provided by Nutritional biochemicals corporation. 2-C^{14} Thymine, 1 μc./μM of specific activity, was provided by Voik radiochemical company. Dicyclohexylammoniumhydrogenphosphate is Prepared with cyclohexylamine and Potassium phosphate.

Preparation of Thymidine Phosphorylase—The thymidine phosphorylase was prepared by the method of Friedkin *et al.* (3) from the water extract of cattle liver.

Measurement of Enzyme Activity—0.1 *M* phosphate buffer 0.2 ml. which containing 6 μM of thymidine, and 0.2 ml. of enzyme solution were incubated in 37° water bath at 60 minutes, the reaction was stopped by the addition of 4 ml. of 0.3 *N* NaOH. The amounts of released thymine was measured spectrophotometrically. One enzyme unit is defined as the enzyme activity which can released 1 μM of thymine per mg. of enzyme protein per hour.

Preparation of C^{14} Labeled Thymidine—C^{14} thymine, 50 to 100 μM (10 to 20 μc.), equall amount or two fold amount of thymidine, 200 μM of dicyclohexylammoniumhydrogenphosphate, 5 ml. of 0.1 *M* phosphate buffer pH 7.0 and thymidine phosphorylase (28 enzyme unit. 30 to 40 mg.), final volume was 5 to 10 ml., were incubated in 37° bath. After appropriately time interval, the reaction was stopped by heating in boiling water bath at 5 minutes, and the denaturated protein was removed by centrifugation. The supernatant adjusted to pH 8.2 and inorganic phosphate was removed by addition of $Ca(OH)_2$ solution.

Cation was removed with Amberlite IR 120 by batch method, then the C^{14} compounds were separated by paper and column chromatography.

Paper Chromotography—The modified solvent of Prusoff *et al.* (9) was used, as the original solvent was suitable for the separation of azathymidine derivatives but was not so effective for the separation of thymidine derivatives. The modified solvent was composed of phosphate buffer of 1/20 *M* (pH 6.0) which saturated with ethylacetate. After upward development, the spots were detected by ultravioletphotography and radioautography.

The radioactive substances were eluted from the paper with 0.1 *N* ammonia.

Ion Exchange Chromatography—The above mentioned reaction mixture, the inorganic phosphate of which was removed previously, was adjusted to pH 10 by addition of concentrated ammonia and adsorbed completely to the column.

After washing with water, the nucleosides were eluted either with 0.01 *M* ammonia formate buffer (pH 8.95) or with 0.1 *M* ammonia acetate buffer (pH 8.95) and collected as each 10 ml. with fraction collector. (Toyo Filter Paper Co., Ltd.).

The fractioned C^{14} compounds were recovered from the buffer solution according following method. Each separated fraction was collected and concentrated to a few ml. by vacum distillation.

To this concentrate was added 10 volume of *n*-butylalcohol, and the solution was mixed by the same volume of diethylether.

The resultant two layers were separated by centrifugation.

The upper layer was ether-*n*-butylalcohol layer containing base or nucleosides, but the lower layer contained only inorganic salts.

The upper layer was concentrated under vacum distillation, and the base or nucleosides were crystallized from diethylether.

Measurement of Ultraviolet Absorption—The amount of thymidine was measured spectrophotometrically by the use of Beckman type spectrophotometer (Hitachi Co., Ltd. type EPU).

The millimolor extinction value for thymidine was adopted as $E_{265}=8500$ in alkali and $E_{265}=9800$ in acid, and for thymine as $E_{300}=3600$ in alkali.

Measurement of Enzyme Protein—The concentration of enzyme was determined according to Kalckar (*10*) by difference of extinction value at 260 mμ and 280 mμ.

Radioactivity Measurement—Each 0.5 ml. of fractionated solution was dried under infrared lamp, and the radioactivity of C^{14} compound was measured by a gas flow proportional counter (Nuclear Measurement Co. Ltd.) under Q gas atmosphere.

RESULTS

Preparation of Thymidine Phosphorylase—By the combination of ammonium sulfate and ethylalcohol fractionation, about 10 fold purification was obtained as shown in Table I.

TABLE I

Cattle Liver Thymidine Phosphorylase

Fraction	Volume	Protein (*mg./ml.*)	Specific activity
Water extract	3500	25.2	0.26
Ammonium sulfate fraction	1350	13.2	0.50
Ammonium sulfate supernatant	3000	5.8	0.11
Ethylalcohol fraction	30	38.3	2.82

Paper Chromatography—By development with modified solvent upward, the reproducible separation was obtained with R_f value of thymidine 0.85 and of thymine 0.70, on the other hand by original solvent the R_f value of thymidine 0.25 and of thymine 0.15. The upward development was advantageous for the separation of relatively large volume of sample, namely 20 to 30 μM of sample was chromatographied satisfactory.

In Fig. 1 is illustrated an example of ultravioletphotogram and in Fig. 2 is illustrated a radioautogram of paper chromatogram.

Ion Exchange Chromatography—Either with amonium acetate buffer or with ammonium formate buffer, thymidine was eluted at first and thymine was followed. But it was well separated with later buffer. (Figs. 3 and 4).

In either case, the peak of radioactivity was preceeded to the peak of ultraviolet absorption. It was probably assumed a kind of isotopic effect.

Incubation period—Friedkin *et al.* (*4*) incubated C^{14} thymine and equimolar thymidine at 7 hours with horse liver enzyme. But when we incubated C^{14} thymine with equimolar thymidine at 18 hours with cattle liver enzyme, the obtained amount of synthesized C^{14} thymidine was about as a half of thymine, although the specific activity of thymidine was equall to the value of thymine. The time factor of enzymatic synthesis of C^{14}

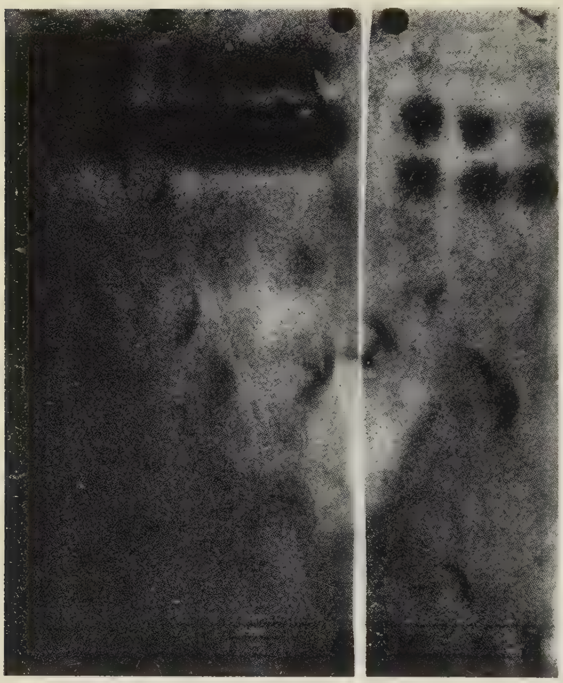

FIG. 1. Ultravioletphotogram of thymine and thymidine.

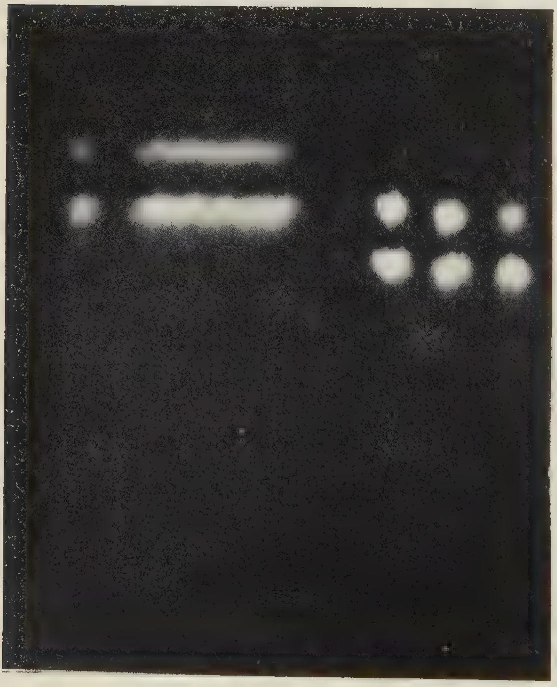

FIG. 2. Radioautochromatogram of thymine C^{14} and thymidine C^{14}. Solvent; 1/20 *M* of phosphate buffer (pH 6.0) saturated with ethylacetate. Development; upward 18 hours.

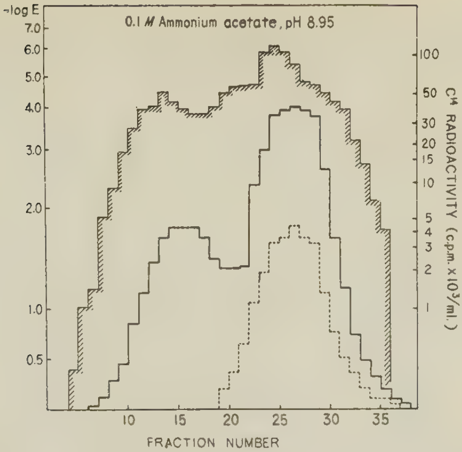

FIG. 3. Separation of C^{14} thymine and C^{14} thymidine by ammonium acetate system chromatography. 50 μM of C^{14} thymine and 100 μM of thymidine were incubated at 18 hours with cattle liver thymidine phosphorylase.

Dowex 1 column, acetate form, 2.5 cm² × 10 cm. long. Elution; 0.1 *M* ammonium acetate buffer (pH 8,95).

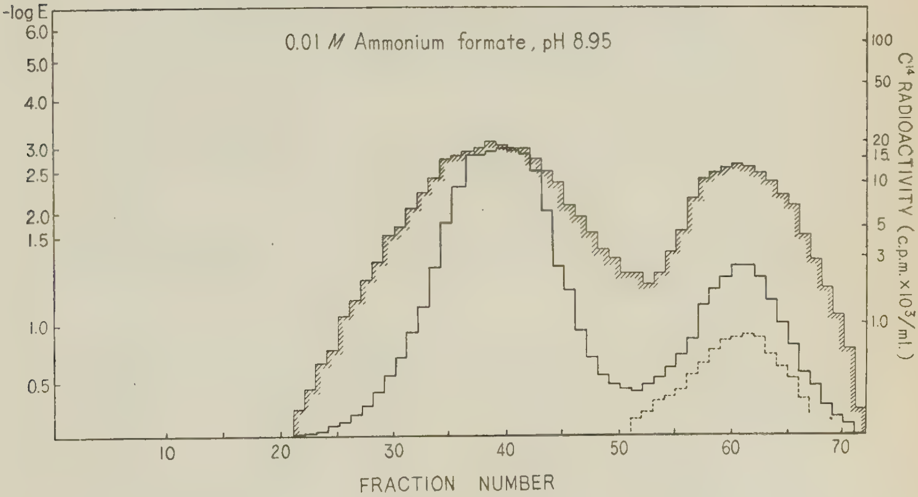

FIG. 4. Separation of C^{14} thymine and C^{14} thymidine by ammonium formate system chromatography. 50 μM of C^{14} thymine (10 μc.) and 100 μM of thymidine were incubated at one hour with thymidine phosphorylase, and 90 μM of C^{14} thymidine was recovered. Elution; 0.01 *M* ammonium formate buffer (pH 8.95).

thymidine was examined either with ammonium sulfate or ethylalcohol fractionated enzyme. C^{14} thymine and equimolar amount of thymidine were incubated with above mentioned enzyme, and the reaction were stopped at

30, 60, 90, 120, 150, 180 minutes and 18 hours after incubation respectively. C^{14} thymine and C^{14} thymidine were separated by paper chromatography, and each specific activity was determined (Fig. 5).

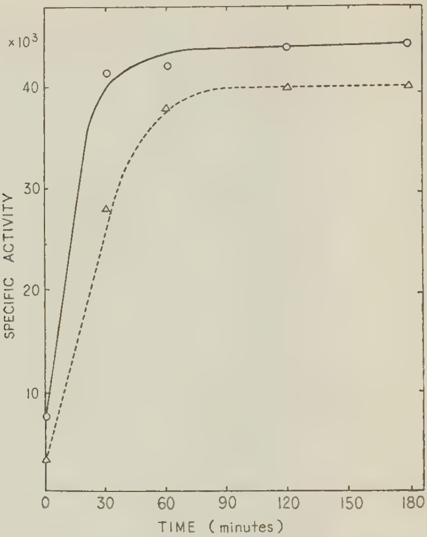

FIG. 5. Time course of enzymatic synthesis of C^{14} labeled thymidine with cattle liver thymidine phosphorylase. ---△---; ammonium sulfate fractionated enzyme, ---○---; ethylalcohol fractionated enzyme.

TABLE II

Time Course of C^{14} Labeling of Thymidine by Thymidine Phosphorylase from Cattle Liver.

Incubation time (*hour*)	3		18	
	Thymidine	Thymine	Thymidine	Thymine
Radioactivity (*counts/min.*)	9360	15360	5760	11904
Amount (μM)	0.096	0.15	0.047	0.105
Thymidine/Thymine (*% of amounts*)	64		45	
Specific activity (*c.p.m./μM*)	97.500	102.400	123.00	114.000

The radioactive thymidine was synthesized rapidly after 30 minutes incubation, the specific activity of thymidine attained nearly the same value of the thymine.

But the amount of thymidine was decreased gradualy by incubation, and after 3 hour incubation the amount was decreased to 64 per cent of the amount of thymine, and after 18 hours to 45 per cent of that of thymine. The prolonged incubation being founded not advantageous, the incubation for one was adopted.

The highest specific activity of C^{14} thymidine thus obtained was 28×10^4 c.p.m./μM.

SUMMARY

The specific activity, 28×10^4 c.p.m./μM, of thymidine was synthesized enzymatically from C^{14} thymine and thymidine with thymidine phosphorylase from cattle liver. C^{14} thimine and C^{14} thymidine were separated either with paper chromatography with $1/20\ M$ of phosphate buffer system, or column chromatography with $0.01\ M$ ammonium formate system.

The enzymatic synthesis of thymidine was so rapid that the specific activity of C_{14} thymidine was nearly equall to that of C^{14} thymine at 30 minutes after incubation, and the prolonged incubation resulted the decrease of the amount of C^{14} thymidine.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

CRYSTALLINE BACTERIAL PROTEINASE

VII. DENATURATION OF BACTERIAL PROTEINASE AND ITS COMPLEX WITH DIISOPROPYL FLUOROPHOSPHATE, AND THEIR OPTICAL ROTATIONS

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(Received for publication, April 24, 1959)

In previous papers (*1*, *2*), it has been reported that bacterial proteinase from *Bac. subtilis*, *N′* (BPN′) reacts stoichiometrically with diisopropyl fluorophosphate (DFP), resulting in inhibition of both its proteolytic and esterolytic activities. The reaction product of BPN′ with DFP has been isolated in a stable crystalline form (*1*). Since it was considered that DFP reacted with chymotrypsin to form a complex similar to the enzyme-substrate complex (*3*, *4*), further knowledge about the relation between the structure and activity of the enzyme might be obtained from an investigation of the difference in structure of BPN′ and its complex with DFP. If it is assumed that the inactivation of an enzyme by denaturing agents such as urea, heat and acid, is caused by deformation of the secondary structure of the enzyme protein, and that native protein is not digested by BPN′, the "ratio of denaturation" of an enzyme protein can be measured by its increase in susceptibility to proteinase. In fact, it was confirmed that the susceptibility of some enzyme proteins to the proteinase increases with decrease in enzymic activity during urea, heat and acid denaturation (*5–9*). It has been found that a transition from the native to the denatured state of proteins is accompanied by an increase in negative specific rotation (*10*), and that in the one-term Drude equation of rotatory dispersion, the value for the dispersion constant is shortened on denaturation (*11*). Moreover, it was found that the rotation and the dispersion constant of proteins are closely related to the helical structure (*12–13*).

In the present investigation a comparison is made of DFP-inhibited BPN′ and BPN′ under various denaturing conditions, using the proteinase method (*5–9*). The possible correlation between their susceptibility to proteinase under above conditions and their rotatory properties was investigated.

Results showed that DFP-inhibited BPN′ appears to be more stable than BPN′ to treatment by various denaturing agents. However there is no difference in the dispersion constants of the two compounds. Results showing that the protein has no bisulfide-linkage contributing to maintenance of the rigid structure of protein, will also be reported here.

MATERIALS AND METHODS

Preparation of the Enzyme Solutions—BPN′ and DFP-inhibited BPN′ were prepared as described in the previous papers (1, *14*) and used after two or more recrystallizations. BPN′ was dissolved in 0.1 *M* ammonium phosphate (pH 7.5). DFP-inhibited BPN′ was suspended in 0.1 *M* ammonium phosphate, the suspension was adjusted with 1 *N* ammonia to pH 9.5, and after complete dissolution the pH was adjusted to 7.5 with 1 *N* phosphoric acid. After centrifugation the supernatants were used for experiments. Protein concentration was determined from the optical density at 280 mμ (*1*). Crystalline trypsin was obtained commercially from the Mochida Pharm. Co., Ltd.

Proteinase of *Streptomyces griseus* was supplied from the Scientific Research Institute Ltd. They were dissolved in 0.1 *M* ammonium phosphate (pH 7.5). The concentration was determined by the dry weight.

Urea Denaturation—The method developed by Okunuki *et al.* (*5–9*) was modified as follows. DFP-inhibited BPN′ and BPN′ were mixed with 12 *M* urea in 0.1 *M* ammonium phosphate (pH 7.5) to final urea concentrations of 8, 6 and 4 *M*. The final concentration of BPN′ in the mixture was 0.1 per cent and that of DFP-inhibited BPN′ was 0.2 per cent. The mixture were incubated at 30° and samples were removed at intervals. With BPN′, 2 ml. of the incubation mixture was added to an equal volume of 1.2 *M* trichloroacetic acid (TCA). With DFP-inhibited BPN′, 1 ml. of the mixture was incubated with 2 ml. of 0.025 per cent BPN′ at 30° for 20 minutes first, and then 1 ml. of 2.4 *M* TCA was added to the solution. The solutions were filtered and the optical density of the filtrates was measured at 280 mμ.

Heat Denaturation—0.1 per cent BPN′ and 0.2 per cent DFP-inhibited BPN′ were incubated at 56° or 60°. At intervals 2 ml. of heat-treated BPN′ solution was removed and mixed with 2 ml. of 1.2 *M* TCA. One millilitre of heat-treated DFP-inhibited BPN′ was added to 2 ml. of 0.025 per cent BPN′ solution, and incubated at 30° for 20 minutes after which 1 ml. of 2.4 *M* TCA was added. Following procedures were the same as for urea denaturation. When other proteinases (*e.g.*, trypsin and the proteinase of *St. griseus*) were used in place of BPN′ to digest the denatured protein, the procedure was the same as for DFP-inhibited BPN′, except that the concentration of both BPN′ and DFP-inhibited BPN′ was 0.2 per cent.

Acid denaturation—0.2 per cent solutions of each protein were prepared by dissolving them into a mixture of equal volumes of 0.02 *M* ammonium phosphate and 0.02 *M* ammonium acetate and adjusting the pH to 4.0 with phosphoric acid solution. These were incubated at 30°. One millilitre of the reaction mixture was removed at suitable intervals, and added to 2 ml. of 0.025 per cent BPN′ in 0.1 *M* ammonium phosphate (pH 7.5.). The mixture was incubated at 30° for 20 minutes at pH 7.4. To terminate the enzymic reaction, 1 ml. of 2.4 *M* TCA was added to the mixture. Following treatments were the same as for urea denaturation.

Determination of Susceptibility—The degree of susceptibility was calculated from the following equation (*5*).

Degree of susceptibility $= \frac{(\mathrm{Etest.} - \mathrm{Einitial}) \times 100}{\mathrm{Efinal} - \mathrm{Einitial}}$ where Einitial is the optical density of the TCA-filtrate at 280 mμ, when the reaction mixture of BPN′ is added to TCA at the start of reaction. That of DFP-inhibited BPN′ is the optical density after incubation with BPN′ for 20 minutes at 30° and then addition of TCA. Etest. is the optical density after the reaction, Efinal is the optical density after complete denaturation of protein by boiling the reaction mixture of BPN′ at 100° for 10 minutes and then addition of TCA, or for DFP-inhibited BPN′ completely denatured, after incubation with BPN′ and then addition of TCA.

Measurement of Optical Rotation—Crystalline BPN′ and DFP-inhibited BPN′ were dissolved in 0.1 *M* and 0.01 *M* phosphate buffer (pH 7.5), and dialyzed overnight against the same buffer in a refrigerator (5°). After centrifugation, the optical rotation of each dialysate was measured at 10° in a Rudolph photoelectric polarimeter, model 200, using a mercury lamp.

RESULTS AND DISCUSSION

Urea Denaturation—It is difficult to estimate the susceptibility of denatured BPN′ to proteinase, since it autolyses even in the presence of as high a concentration of urea as 8 *M* (Fig. 1).

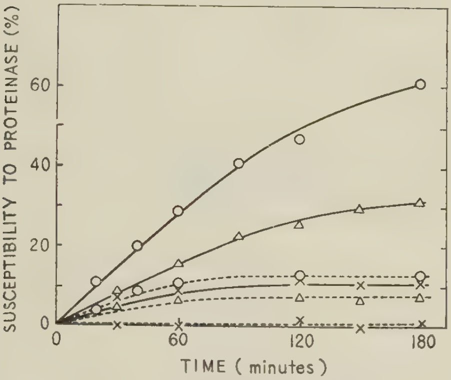

FIG. 1. Susceptibility of BPN′ and DFP-inhibited BPN′ to BPN′ during treatment with urea. 0.2 per cent DFP-inhibited BPN′ and 0.1 per cent BPN′ were incubated with 8, 6 and 4 *M* urea at 30°. BPN′: —○—, in 8 *M* urea; —△—, in 6 *M* urea; —×—, in 4 *M* urea. DFP-inhibited BPN′: --○--, in 8 *M* urea; --△--, in 6 *M* urea; --×--, in 4 *M* urea.

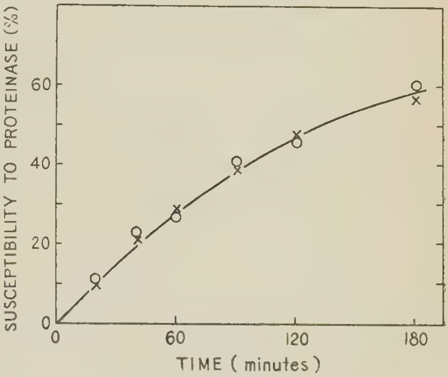

FIG. 2. Autodigestion of BPN′ in 8 *M* urea. 0.1 per cent BPN′ was incubated with 8 *M* urea. —×—: Reaction mixture was incubated with 0.1 per cent BPN′ before the addition of TCA. —○—: Reaction mixture was mixed with TCA without incubation with BPN′.

For this reason the degree of autodigestion was adopted as a criterion of the degree of susceptibility to urea denaturation. This criterion could be dubious for the following reasons: (i) The rate of inactivation by urea may inhibit autodigestion of BPN′, so that in the presence of urea, autodigestion may be imcomplete. (ii) The concentration (0.017 per cent) of proteinase used to digest the denatured DFP-inhibited BPN′ is much lower than that of BPN′ (0.1 per cent) in the autodigestion, so that the digestion of DFP-inhibited BPN′ by such a lower concentration of proteinase may not be complete during the 20 minutes incubation time. (iii) Autodigestion of BPN′ is carried out in 8 *M* urea while the digestion of DFP-inhibited BPN′ is carried out at a lower concentration (2.7 *M*) of urea. Therefore denaturation of DFP-inhibited BPN′ may be reversed at a lower urea concentration. In order to examine the above possibilities, the following experiments were made. (i) 0.1 per cent BPN′ was incubated with 8 *M* urea. At intervals, 2 ml. of the reaction mixture was added to 2 ml. of 1.2 *M* TCA. Simultaneously another 2 ml. of the reaction mixture was mixed with 1 ml. of 0.1 per cent BPN′, and incubated at 30° for 20 minutes before the addition of 1 ml. of 2.4 *M* TCA. No difference in optical density in the two systems (Fig. 2). Therefore there is enough BPN′ to digest the denatured BPN′ completely during the reaction period. (ii) The time course of digestion of denatured DFP-inhibited BPN′ was investigated. As shown in Fig. 3, digestion by 0.017 per cent BPN′ was complete within 20 minutes. (iii) 0.2 per cent DFP-inhibited BPN′ was incubated with 8 *M* urea. One millilitre of the reaction mixture was added to 2 ml. of 0.025 per cent BPN′ solution containing 8 *M* urea, while the same aliquot of the mixture was added to 2 ml. of BPN′ solution containing no urea. It was found that the rate of digestion was not affected by the urea concentration (Fig. 4).

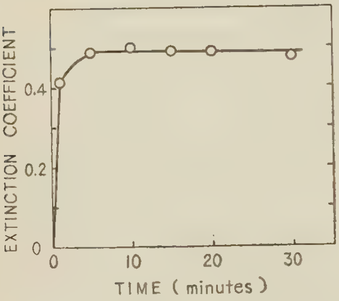

FIG. 3. Digestion of denatured DFP-inhibited BPN′. DFP-inhibited BPN′ denatured by boiling for 10 minutes was digested with 0.017 per cent BPN′.

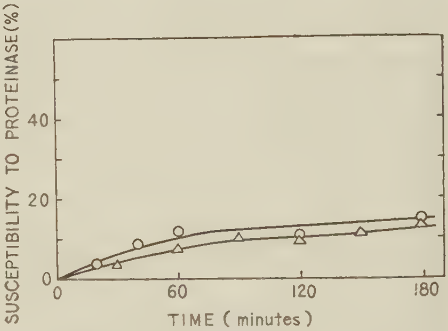

FIG. 4. Susceptibility of DFP-inhibited BPN′ to BPN′. 0.2 per cent DFP-inhibited BPN′ was incubated with 8 *M* urea. —△—: Reaction mixture was digested by BPN′ containing 8 *M* urea. —○—: Reaction mixture was digested by BPN′ containing no urea.

From the above results it was concluded that this modification is valid. Thus to ascertain the difference in susceptibility of BPN′ and DFP-inhibited BPN′ to urea denaturation, their susceptibilities to BPN′ were determined in various urea concentrations (Fig. 1).

It was found that DFP-inhibited BPN′ had a lower susceptibility to the proteinase than BPN′ at various urea concentrations.

Heat Denaturation—Heat denaturation was carried out at 60°. In heat denaturation, autodigestion of BPN′ was adopted as the criterion of susceptibility to proteinase. BPN′ was more susceptible to the proteinase than

DFP-inhibited BPN′ (Fig. 5). However, as DFP-inhibited BPN′ treated at 60° was incubated with BPN′ at 30° for digestion, its denaturation may already

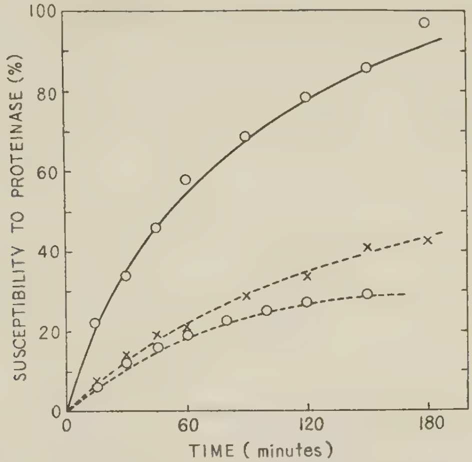

FIG. 5. Susceptibility to BPN′ on heat treatment. —○—: BPN′ was autodigested at 60°. --×--: DFP-inhibited BPN′ treated at 60° was digested by BPN′ at 60°. --○--: DFP-inhibited BPN′ treated at 60° was digested by BPN′ at 30°.

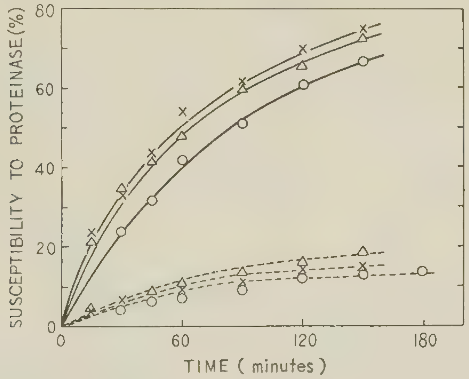

FIG. 6. Susceptibility to proteinase on treatment at 56°.

--×--: DFP-inhibited BPN′ digested by trypsin.
— × —: BPN′ digested by trypsin.
--△--: DFP-inhibited BPN′ digested by the proteinase of *St. griseus*.
— △ —: BPN′ digested by the proteinase of *St. griseus*.
--○--: DFP-inhibited BPN’ digested by BPN′.
— ○ —: BPN′ autolysis.

have been reversed by the time of digestion, while BPN′ have been autodigested at 60°. Thus DFP-inhibited BPN′ treated at 60° was incubated with BPN′ at 60° and 30°. DFP-inhibited BPN′ incubated at 60° with BPN′ was digested to a certain extent than at 30°, although the degree of digestion was much lower than that of BPN′ (Fig. 5). Heat denaturation at 56° was also carried out with other proteolytic enzymes (trypsin and protease of *St. griseus*) and the same results were obtained as with BPN′ (Fig. 6).

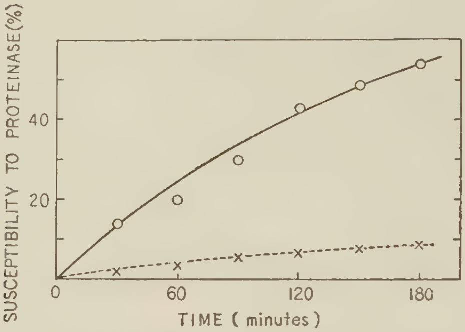

FIG. 7. Susceptibility to BPN′ on acid treatment (pH 4). —○—: BPN′ incubated at pH 4 was neutralized to pH 7.5, and incubated with BPN′. --×--: DFP-inhibited BPN′ incubated at pH 4 was neutralized to pH 7.5, and incubated with BPN′.

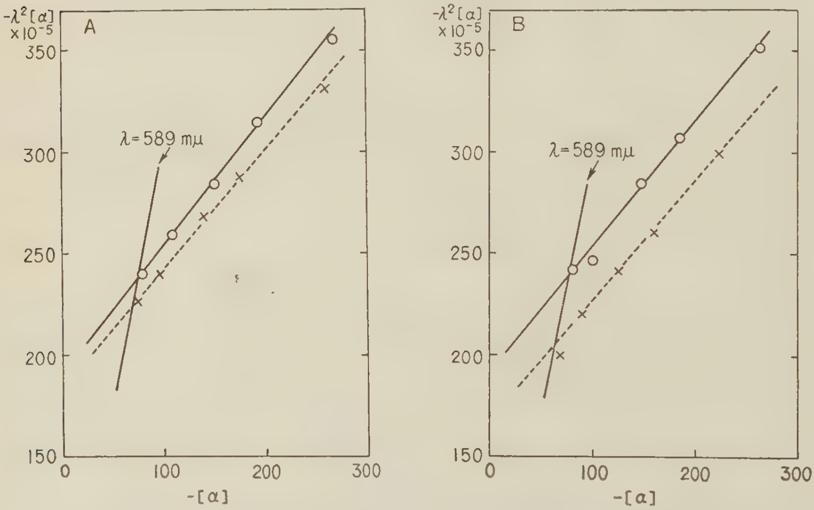

FIG. 8. Dispersion plot of DFP-inhibited BPN′ and BPN′.
A: Conditions as in A in Table I.
—○—; DFP-inhibited BPN′; --×--; BPN′.
B: Conditions as in B in Table I.
—○—; DFP-inhibited BPN′; --×--; BPN′.

Acid Denaturation—The susceptibility of DFP-inhibited BPN′ to BPN′ was lower than that of BPN′ on incubation under acidic condition (pH 4) at 30° (Fig. 7). DFP-inhibited BPN′ was more stable than BPN′ to denaturation by urea, acid and heat. This suggests that they have different structures. The results might be due to steric hindrance in DFP-inhibited BPN′ by combination with DFP. However since other proteinases with different specificity used in heat denaturation gave the same results, it is more reasonable to consider that there is a difference in secondary or tertiary structure.

Rotatory Properties—The dispersion constant and the specific rotation at at 589 mμ of BPN′ and DFP-inhibited BPN′ are presented in Table I and Figs. 8-A and 8-B.

TABLE I

Dispersion Constant and Specific Rotations at 589 mμ of BPN′ and DFP-inhibited BPN′

Sample	Dispersion Constant	Specific Rotation at 589 mμ
BPN′	A 246 mμ	−63.4°
	B 243	−57.7°
DFP-inhibited BPN′	A 247	−67.6°
	B 247	−67.2°

A: One per cent BPN′ and DFP-inhibited BPN′ were dialyzed overnight against 0.1 *M* ammonium phosphate (pH 7.5), and after centrifugation the optical rotations were measured at 10°.

B: 0.5 per cent BPN′ and DFP-inhibited BPN′ were dialyzed overnight against 0.01 *M* ammonium phosphate (pH 7.5), and their optical rotations were measured at 10°.

The values for the dispersion constants were the same within the limits of experimental error. If the value of the dispersion constant is related to the amount of the helical component, this suggests that there was no difference in helical structure.

However from the specific rotation at 589 mμ, it may be suggested that BPN′ has a slightly larger helical component than DFP-inhibited BPN′, assuming that an increase in levorotation indicates denaturation by unfolding of the helical structure of protein. This difference however may not be caused by the helical structure but by intrinsic rotation of amino acid residues. The slight change in specific rotation may also result from an unknown factor which is responsible for the stability of DFP-inhibited BPN′. Further investigations on moderate intramolecular changes will be needed to elucidate this point.

Amino Acid Analysis—The amino acid composition of DFP-inhibited BBN′ was analyzed by the method of Stein and Moore (*15*)*.

The results were given in Table II, in which the molecular weight of the protein is assumed to be 30,500 (*16*). It is of interest that the protein has no cystine residue, though 0.3 *M* of cysteinic acid was found.

TABLE II

Amino Acid Analysis of DFP-inhibited BPN′

Amino acid[1)]	Ext. 1	Exp. 2	Amino acid	Exp. 1	Exp. 2
Glycine	30.7		Serine	31.7	32.5
Alanine	34.9		Threonine	11.5	11.9
Valine[2)]	27.2	21.9	Cy-SO_3H	0.31	
Leucine	15.3	14.2	Arginine	4.87	
Isoleucine	11.7	10.7	Histidine	2.22	2.23
Proline		17.0	Iysine	6.54	6.04
Phenylalanine	3.46	3.45	Aspartic acid	27.7	28.0
Tyrosine		9.00	Glutamic acid	(14.6)	13.1
Tryptophan		3.88	NH_3	(45.5)	

1) The value is the number of moles of amino acid per mole of protein.

2) An inexplicable difference in value was found in two experiments.

This result was confirmed by amperometric titration of BPN′**, and by the hydrazynolysis method***, which gave a value of 0.3 mole of cysteine- and cystine-*S* per mole protein.

SUMMARY

1. The behavior of BPN′ and DFP-inhibited BPN′ were compared on denaturation by urea, heat and acid, and their optical rotations were measured. DFP-inhibited BPN′ was more stable towards denaturing agents than BPN′ but no difference in dispersion constant was found.

2. The amino acid composition of DFP-inhibited BPN′ was determined. The results showed that this enzyme protein has no bisulfide-linkage.

3. The relation between the susceptibility of BPN′ and DFP-inhibited BPN′ to proteinases during denaturation, and the optical rotatory properties is discussed.

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* We were indebted to Dr. A. Tsugita and Mr. Y. Murakami of Akabori laboratory of Osaka University for this analysis.

** Unpublished datum of T. Yonetani in our laboratory.

*** For the analysis we were indebted to Miss T. Fujiwara of Akabori laboratory.

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The Journal of Biochemistry, Vol. 46, No. 11, 1959

STUDIES ON THIAMINOKINASE FROM BAKER'S YEAST*

I. PURIFICATION AND PROPERTIES

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(Received for publication, May 8, 1959)

Enzymatic phosphorylation of thiamine has clearly been established as a prerequisite for its participation as a coenzyme with many important reactions catalyzed by pyruvic and α-ketoglutaric dehydrogenase, carboxylase, transketolase, and others. The enzyme thiaminokinase has, therefore, been studied by many investigators and partially purified from tissues of mammalian species and also from extracts of yeast.

Yeast thiaminokinase was first prepared by Weil-Malherbe (*1*) from Lebedew juice of dried brewer's yeast, and it was shown that ATP** was required as phosphate donor in this reaction. Later studies were made by Thoai and Chevillard (*2*), and by Steyn-Parvé (*3*), and a partially purified protein fraction was obtained from baker's yeast by the latter. These studies were made with a crude system and much remained to be studied of the properties and mechanisms of the reaction. As for the enzymes from animal tissues, the investigation of Leuthardt and Nielsen (*4*) was reported, and more recent studies will be published by Mano and Tanaka (*5*) in this laboratory.

The work of Weil-Malherbe and subsequent studies by Leuthardt and Nielsen suggested a direct pyrophosphate transfer from ATP to thiamine, TMP being less active than thiamine, and the reaction mechanism was postulated as shown in the following equation, without any conclusive evidence.

$$\mathrm{T+ARPPP \longrightarrow TPP+ARP}$$

The present work was started with two purposes. First, it was intended to prove the mechanism of transpyrophosphorylation with an essentially pure enzyme. Second it seemed of interest to study nucleotide specificity of the enzyme, as the present author found in the course of a preliminary study that the nucleotide specificity of this enzyme is quite dependent upon experimental conditions under which the reaction is carried out, *i.e.*, the metal concentrations and/or pH.

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* This work was supported in part by Grant-in-Aid for Fundamental Scientific Research from the Ministry of Education.

** The following abbreviations are used: ATP or ARPPP, adenosine triphosphate; ADP, adenosine diphosphate; AMP or ARP, adenosine monophosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; IDP, inosine diphosphate; DPN, diphosphopyridine nucleotide; TTP, thiamine triphosphate; TDP or TPP, thiamine diphosphate; TMP, thiamine monophosphate; NPT, neopyrithiamine; OT, oxythiamine; ET, ethylthiamine; BT, butylthiamine; OMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine; Th, 4-methyl-5-hydroxyethylthiazole; Tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose diethylaminoethyl-cellulose; EDTA, ethylenediamine tetraacetate; and NDPkinase, nucleoside diphosphokinase.

The present paper describes the purification and some of the properties of yeast thiaminokinase. Preliminary reports of the nucleotide specificity (*6*) and reaction mechanism (*7*) were presented elsewhere, the details of which will be treated in the succeeding paper (*6a*).

EXPERIMENTAL

Materials—Nucleoside polyphosphates, DPN, and NPT were purchased from the Sigma Chemical Company and from the Pabst Laboratories. DPN, 92 per cent pure, was reduced chemically with dithionite (*8*). Thiamine, TMP, TDP, OT, ET, BT, OMP, and Th were supplied by the Takeda Pharmaceutical Industries, Ltd., Japan. TTP was synthesized by the procedure of Viscontini *et al.* (*9*) and purified by column chromatography as will be described later. Phosphoenolpyruvate prepared by the method of Baer (*10*), was kindly furnished by Miss S. Washio. DEAE-cellulose used in the early experiments was kindly given by Dr. K. Yamada, and for the further studies the preparation was synthesized according to Peterson and Sober (*11*). ATP labeled in the two terminal phosphorus atoms was prepared by the method of Tatibana *et al.* (*12*).

Enzymes—Apocarboxylase was prepared from baker's yeast as described previously (*13*). Crystalline lactic dehydrogenase was obtained from the Sigma Chemical Company. This enzyme contained a considerable amount of pyruvic kinase and was used as a source of both lactic dehydrogenase and pyruvic kinase.

Determination of Protein Concentration in Enzyme Solution—In crude preparations, protein was measured by biuret color reaction (*14*). Crude extract of dried baker's yeast was used as a standard reference after being checked by nitrogen analysis. After ammonium sulfate fractionation, protein determination was made spectrophotometrically (*15*) and the approximate formula of Kalckar (*16*) was applied. During chromatography, protein was measured by light absorption at 280 mμ.

Determination of TDP—TDP was measured manometrically by carbon dioxide liberation from pyruvate after recombination of the apocarboxylase with TDP (*16a*). The assay mixture contained 250 μ moles of sodium citrate buffer (pH 6.0), 30 μ moles of $MnCl_2$, 200 μ moles of sodium pyruvate, 0.2 ml. of apocarboxylase solution, an appropriate amount of TDP, and water in a total volume of 3.0 ml. The carbon dioxide evolution was measured for 30 minutes at 30°. In some cases, 1.5 μ moles of NPT was added to the system in order to eliminate the thiaminokinase activity as a contaminant in the apocarboxylase preparation. The addition of NPT was usually unnecessary, because, as will be shown later (*6a*), ATP was practically inactive as phosphate donor in the thiaminokinase reaction under above assay conditions. Only when GTP or ITP was used as phosphate donor, the introduction of NPT became indispensable as these nucleotides could phosphorylate thiamine even under these conditions.

Assay for Thiaminokinase—The enzyme was incubated for 60 minutes at 30°, with 1.5 μ moles of thiamine, 1.5 μ moles of ATP, 3 μ moles of $MnSO_4$ and 30 μ moles of

Tris buffer, pH 8.6, and enzyme in a final volume of 1.5 ml. The reaction was terminated by heat inactivation for 2 minutes in a boiling water bath. A suitable aliquot was taken after dilution and the amount of TDP formed was determined manometrically. A blank without ATP was treated in the same manner.

The enzymatic activity was proportional to protein concentration and linear with time under the standard assay conditions (Figs. 1 and 2).

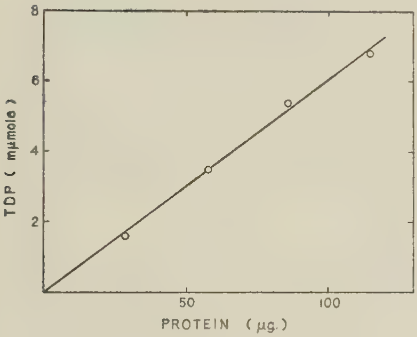

Fig. 1. Influence of enzyme concentration on activity.

Standard conditions were used, and the data are given in mμ moles of TDP formed per 60 minutes.

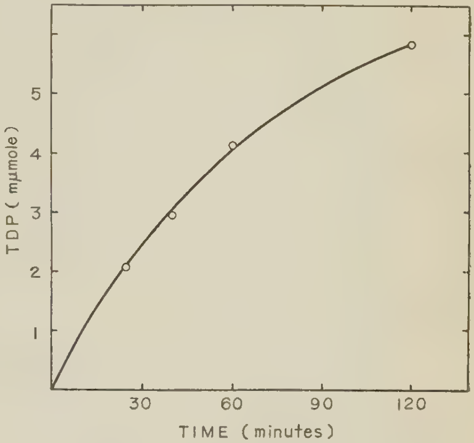

FIG. 2. Time curve of the reaction. Standard conditions were used with the enzyme preparation after ammonium sulfate fractionation.

A unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 mμ mole of TDP, under the above conditions. The specific activity of the enzyme was defined as units per mg. of protein. In some cases enzymatic activity was expressed as μl. of CO_2 evolved for 30 minutes or as mμg. of TDP formed per hour.

Assay for ATPase, Adenylate Kinase, and NDPkinase (17) Activity—During purification of the enzyme, activity of these three enzymes was checked with the use of pyruvic kinase-lactic dehydrogenase system. The reaction mixture was similar to that described by Bucher and Pfleiderer (*18*) as an assay system for pyruvic kinase except that ADP was omitted. After the addition of the protein fraction to be tested, each of the three enzyme activities was measured by the addition of ATP alone, ATP plus AMP, or ATP plus IDP, for ATPase, adenylate kinase and NDPkinase, respectively.

Identification of Reaction Products—Column chromatography on Dowex 1 (Cl form, X-4, 200–300 mesh) was carried out by stepwise elution based on the hydrochloric acid-sodium chloride system according to Cohn and Carter (*19*). Thiamine phosphates were separated by successive elution from the column with 0.001 *N*, 0.002 *N*, and 0.01 *N* HCl, for TMP, TDP (*7*) and TTP, respectively.

Paper chromatography of nucleotides and thiamine derivatives was developed according to Krebs and Hems (*20*) except that *n*-butyric acid was used in place of iso-butyric acid. The R_f values of authentic samples were 0.95, 0.90, 0.87, 0.65, 0.60, 0.52, and 0.48, respectively, for thiamine, TMP, TDP, TTP, AMP, ADP, and ATP. Inorganic phosphate, and nucleoside phosphates of inosine and guanosine had smaller

R_f values than ATP. Thiamine derivatives were also chromotographed by the method of Siliprandi *et al.* (*21*) with a solvent system composed of *n*-propanol, water, and *M* acetate buffer, pH 5.0 (70 : 20 : 10). The R_f values in this system were 0.31, 0.15, and 0.07, for thiamine, TMP, and TDP, respectively. The spots were located on paper by ultraviolet absorption. Thiamine derivatives were also identified by thiochrome reaction (*22*).

RESULTS

(*1*) *Purification of the Enzyme*

Preparation of Crude Extract—100 g. of dried baker's yeast was suspended in 300 ml. of 0.1 *M* disodium phosphate at 37° for 3 hours with occasional shaking, followed by extraction at room temperature for additional 3 hours. The suspension was centrifuged and the supernatant solution was cooled to 2°. The precipitate was washed with 150 ml. of 0.1 *M* disodium phosphate, the washings were combined with the original extract, and the total solution was filtered with suction through a large Buchner funnel employing 50 g. of Hyflo Super-Cel.

Heat Denaturation of Impurities—The filtrate (*ca.* 300 ml.) was immersed in a water bath of 55° and stirred continuously. When the inside temperature had reached 50°, the outside temperature was lowered to the same level and heating was continued for 10 minutes; after cooling, the mixture was centrifuged. From this point all the operations were performed at 0–4°.

Acetone Precipitation—To each 100 ml. of the supernatant solution, 55 ml. of ice-cold acetone was slowly added, while the temperature of the mixture was maintained at about 0°. The resulting precipitate was separated by centrifugation at 0° and discarded. To each 100 ml. of the supernatant solution, 20 ml. of cold acetone was added (36–46 per cent), the temperature being kept at $-2°$. After standing for 15 minutes, the precipitate was obtained by centrifugation at $4{,}500 \times g$ for 10 minutes and suspended in about 20 ml. of cold water, and dialyzed overnight at 0° against distilled water.

Fractionation with Ammonium Sulfate—After dialysis, the precipitate was removed by centrifugation. The supernatant solution was diluted to 200 ml. and 64 g. of solid ammonium sulfate was added slowly under stirring. The precipitate was centrifuged off and discarded. An additional 9 g. of ammonium sulfate was added per 100 ml. of the supernatant solution (0.51–0.61 saturation) and stirring was continued for 20 minutes after the ammonium sulfate had dissolved. The precipitate obtained after centrifugation at $4{,}500 \times g$ for 20 minutes was taken up in a minimal amount of water and dialyzed overnight against 2 liters of 0.05 *M* Tris buffer, pH 7.4.

Chromatography on DEAE-cellulose—Chromatography on DEAE-cellulose was used as the final step of purification. Columns (2.5×15 cm.) were prepared from the adsorbent which had been equilibrated with 0.05 *M* Tris buffer, pH 7.4. The enzyme solution dialyzed overnight against the same buffer was applied to the column and allowed to drain into the column by gravity. The enzyme was completely adsorbed on the column, no activity being observed in the effluent. The column was then washed with 200 ml. of 0.05 *M* Tris buffer, pH 7.4, until the optical density at 280 mμ decreased to about 0.1. A large amount of inactive protein which was not adsorbed on the column was separated in this step.

Then the chromatogram was developed by gradient elution based on the Tris buffer-NaCl system using a mixing vessel containing 200 ml. of 0.05 *M* Tris buffer, pH 7.4 and with 0.4 *M* NaCl in 0.05 *M* Tris buffer, pH 7.4, in the reservoir vessel. The eluate was collected at a rate of 15 ml. per hour in 5-ml. fractions and assyed for protein and enzyme activity. This step may also be performed at pH 8.6.

A typical result of the chromatography is given in Fig. 3. In some cases,

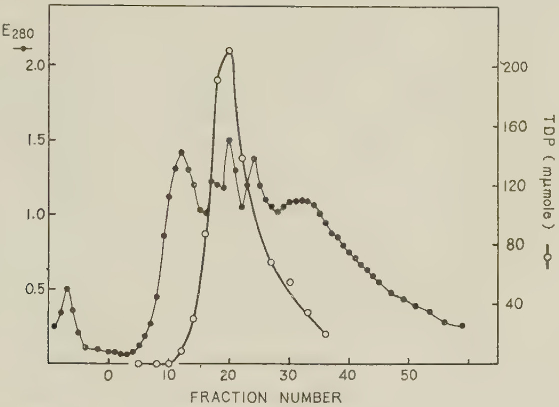

FIG. 3. Column chromatography of the enzyme.

The enzyme after ammonium sulfate fractionation was applied to DEAE-cellulose column (2.5×15 cm.) which had been equilibrated with 0.05 *M* Tris buffer, pH 8.6. The chromatography was performed by gradient elution as described in the text except that pH was 8.6. —●—, E_{280} ; —○—, thiaminokinase activity.

TABLE I

Purification of Thiaminokinase

Fraction	Volume	Activity	Total activity	Protein	Specific activity	Yield
	(*ml.*)	(*units/ml.*)	(*units*)	(*mg./ml.*)	(*units/mg.*)	(*per cent*)
I. Crude extract	510	20.9	10,660	13.9	1.5	100.0
II. Heat treatment	510	18.8	9,600	—	—	—
III. Acetone precipitation	200	41.8	8,360	13.8	3.0	78.4
IV. Ammonium sulfate fractionation	200	37.6	7,520	4.44	8.5	70.6
V. Column chromatography	10	188.0	1,880	1.25	150.5	17.7

the enzyme was further rechromatographed on the column with a similar system at different pH. The adjacent peaks of the above chromatogram were thus separated, but it was impossible to obtain a preparation of higher specific activity, the enzyme being fairly unstable upon purification.

A summary of the typical purification is shown in Table I. With these steps of purification, a 100-fold increase in specific activity was obtained with a yield of about 18 per cent. The purified enzyme contained a little ATPase activity, but neither NDPkinase nor adenylate kinase activity when assayed as described under Experimental.

Properties of the Enzyme—The enzyme solution was clear and had no color. It was kept deep-freezed at −4° and lost only a little activity over a period of 2 months. When stored in a salt-free solution, some precipitation and denaturation occurred.

Stability of the Enzyme—The enzyme in acetate buffer, pH 4.5, and 5.7, and in Tris-maleate buffer, pH 6.2, was immersed in a water bath for 30 minutes at 37°. After removal of the denaturated protein by centrifugation and adjusting pH to 8.6, assay of the enzyme activity, respectively, showed 54, 85, and 100 per cent of the initial values. The same stability test was carried out at 0° with the addition of *M* acetic acid to a final pH of 4.0, 4.25, and 4.5. After being placed in an ice-bath for 5 minutes, the precipitated protein was removed by centrifugation, and the activity of the supernatant solution was found to be 52, 85, and 100 per cent of the initial values, respectively.

(2) *Some Properties of Reaction Catalyzed by Thiaminokinase*

Requirements for Reaction—The maximal rate of reaction was dependent on the presence of ATP, thiamine, divalent cation, and the enzyme (Table II).

TABLE II

Requirements for Thiaminokinase Activity

Additions	CO$_2$ evolved during 30 mins.
	(*μl.*)
Complete system	304.6
,, -ATP	0.9
,, -thiamine	6.7
,, -MnSO$_4$	7.6
,, -enzyme	2.7

The complete system contained: 1.5 μ moles of thiamine; 1.5 μ moles of ATP; 3 μ moles of MnSO$_4$; 30 μ moles of Tris buffer, pH 8.6; enzyme 28.8 μg.; and water to make 1.5 ml. Components were omitted as indicated. Incubation at 30° for 60 minutes.

Identification of TDP—The present assay method was fairly specific for TDP, and thus it clearly indicated the reaction product as TDP. Further identification of TDP was made by paper chromatography and ion-exchange chromatography as follows: After deproteinization with $HClO_4$, the denaturated protein was removed by centrifugation and resulting supernatant was passed through a charcoal column treated with *sec.*-octanol according to Threlfall (*22*). Adenosine and thiamine derivatives were eluted from the charcoal column with 2 ml. of 10 per cent aqueous pyridine. After removing pyridine by distillation under a reduced pressure, the residue was dissolved in a minimal amount of water and submitted to ascending chromatography on Tôyô Roshi No. 53 filter paper with a solvent system of Krebs and Hems. The spot corresponding to TDP was cut off, eluted with dilute HCl and identified by its coenzyme activity using apocarboxylase preparation.

By this paper chromatography, it was impossible to distinguish TMP and TDP clearly. Therefore, in a separate experiment with labeled ATP-P^{32}, the products were submitted to column chromatography on Dowex 1 as described below. The reaction was terminated by heating in a boiling water bath. The precipitated protein was centrifuged, and washed once with a few ml. of water. The combined supernatant was diluted about 6-fold and then applied to Dowex 1 column (1.1×8 cm.). 4 μ moles of non-labeled TMP and TDP were added as carrier. The column chromatography was carried out as described under Experimental. The elution was followed by means of P^{32} analysis and measurement of ultraviolet absorption. Each peak was identified spectrophotometrically and after lyophilization, by paper chromatography. TDP was also identified by its coenzyme activity. The results indicated clearly that the main product of the reaction was TDP, as TMP contained very little radioactivity, and TTP was not detected. In another experiment, 4 μ moles of non-labeled TMP was added before incubation. If the reaction

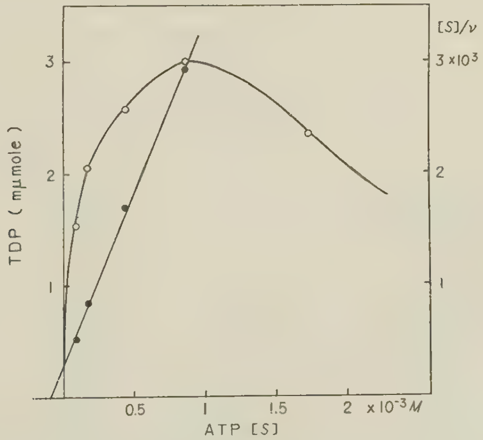

FIG. 4. Dependence of the reaction on the concentration of ATP.

Standard reaction mixture was used except for the concentrations of ATP. Incubation for 20 minutes, at 30°. —○— TDP formed, —●— [*S*]/*v*.

involved the intermediate formation of TMP, a considerable loss in specific activity of TDP would be expected. However, no such dilution was observed. These results leave little doubt that the reaction product is TDP.

Dependence on Substrate Concentration—Figs. 4 and 5 demonstrate the dependence of the reaction rate on the concentrations of ATP and thiamine, respectively. An excess of ATP resulted in an appreciable inhibition of the rate. No inhibition was observed in thiamine concentrations up to 5×10^{-3} *M*. From these figures, Michaelis constants (K_m) of the reaction were calculated

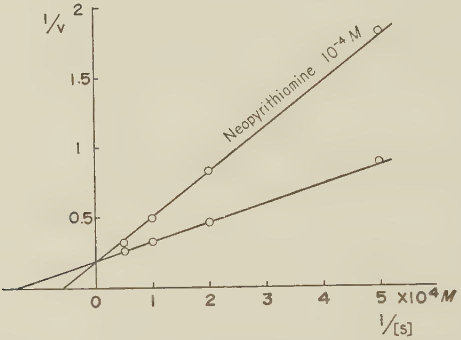

FIG. 5. Dependence of the reaction on thiamine and competitive inhibition of the thiaminokinase by NPT.

Reaction mixture was as described for the standard assay except for thiamine as indicated and the addition of 1×10^{-4} *M* NPT, when present, in a total volume of 1.5 ml., with incubation for 20 minutes at 30°.

TABLE III

Effect of Different Cations on the Reaction

Salt	mμ moles of TDP per hour
$MnSO_4$ (2×10^{-3} *M*)	1.52
$MgSO_4$ (6.7×10^{-3} *M*)	1.08
$CoSO_4$ (,,)	2.16
$CaCl_2$ (,,)	0.24
$NiSO_4$ (,,)	0.23
RbCl (,,)	0.02
$ZnSO_4$ (,,)	0.34
$FeSO_4$ (,,)	0.29
$FeCl_3$ (,,)	0.08

Standard conditions were used except that the pH was 7.0 instead of 8.6. After terminating the reaction, 0.3-ml. aliquots were taken and assayed for TDP. Under these conditions, no inhibition was observed of the apocarboxylase system.

as 1.0×10^{-4} and 0.75×10^{-4} *M* for ATP and thiamine, respectively.

Metal Requirement—The reaction has an absolute requirement for divalent cations. As shown in Table III, Mn^{++}, Mg^{++}, and Co^{++} were most effective, while Ca^{++}, Ni^{++}, Rb^{+}, Zn^{++}, Fe^{++}, and Fe^{+++} showed little or no activity. The latter three ions strongly inhibited the reaction when added with Mn^{++}. These experiments were carried out at pH 7.4 in order to avoid precipitation of some metal oxides at alkaline pH and the concentration of each cation was fixed arbitrarily at 6.7×10^{-3} *M* except for that of Mn^{++}. The relative effectiveness of divalent cations was influenced by various factors such as metal concentrations, pH, buffer system used, and nucleotide concentrations. The dependence of nucleotide specificity on metal concentrations will be described elsewhere (*6*, *6a*).

(*i*) *Optimal Concentrations*: The optimal concentrations of Mn^{++}, Mg^{++}, and Co^{++} were determined at pH 7.0 and pH 8.6 with ATP (1×10^{-3} *M*) as phosphate donor (Fig. 6). As may be seen from the figure, higher concentrations of Mn^{++} and Co^{++} ions were inhibitory at pH 8.6 while at pH 7.0 no inhibition occurred with an excess of either of the three cations.

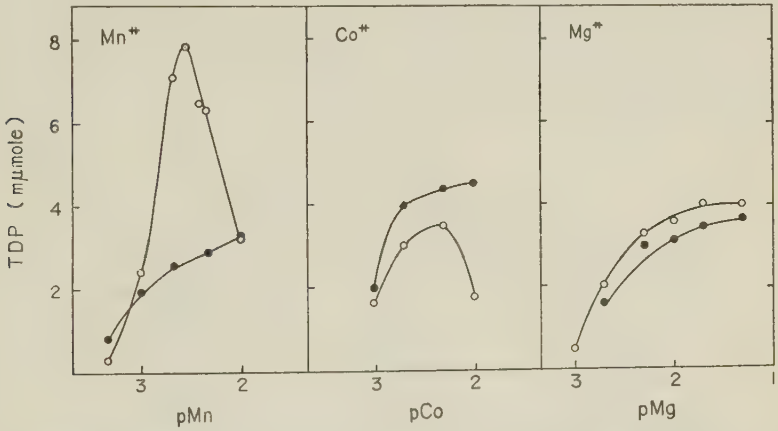

FIG. 6. Effect of metal concentration on thiaminokinase reaction. Standard conditions were used except for the metal concentrations and the pH as indicated. —○— pH 8.6, —●— pH 7.0.

(*ii*) *Comparative Effect of Mn^{++}, Mg^{++}, and Co^{++}*: The effect of Mn^{++}, Mg^{++} and Co^{++} on the rate of reaction was compared at each optimum concentration. The relative activity ratio of three metal ions markedly differed with pH (Fig. 7).

(*iii*) *Relation to Nucleotide Concentrations*: As shown in Fig. 8, the optimal concentration of Mn^{++} was dependent on the concentration of ATP.

pH Optimum of the Enzyme—Otimal pH of the enzyme was shown to differ with the species and concentrations of divalent cations. Fig. 7 demonstrates the pH-activity curves with Mn^{++} (0.003 *M*), Mg^{++} (0.05 *M*), and Co^{++} (0.005 *M*). The concentration of each metal was chosen based on the data given in Fig.

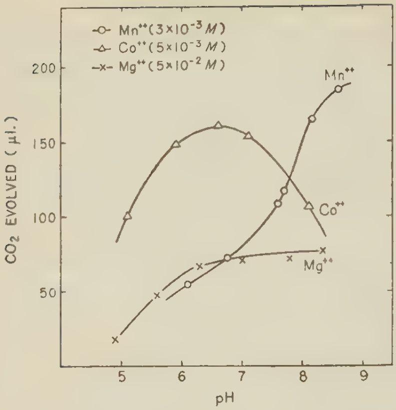

FIG. 7. Thiaminokinase activity as a function of pH.

The reaction mixture contained: thiamine, $1\times10^{-3}\,M$; ATP, $1\times10^{-3}\,M$; divalent cation as indicated; Tris-maleate buffer, $0.02\,M$, pH as specified; enzyme; and water to final volume of 1.5 ml. —○— Mn^{++}, $3\times10^{-3}\,M$; —△— Co^{++}, $5\times10^{-3}\,M$; —×— Mg^{++}, $5\times10^{-2}\,M$.

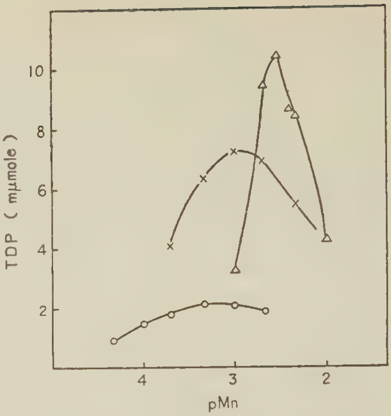

FIG. 8. Dependence of the optimum concentration of Mn ions on the concentration of ATP.

Standard conditions were used except for the concentration of Mn ions and of ATP as indicated. The data are given in mµ moles of TDP formed per 60 minutes. —△— $1\times10^{-3}M$ ATP; —×— $1\times10^{-4}M$ ATP; —○—, $1\times10^{-5}M$ ATP.

TABLE IV

Substrate Specificity of Thiaminokinase

Phosphate donor	Phosphate acceptor	TDP formed
		(*µl.* CO_2 *per 30 mins.*)
ATP	Thiamine	160.0
ADP	Thiamine	1.3
ATP	TMP	15.0

Standard conditions were used except for the substrate as indicated. Concentrations of phosphate acceptors and donors were $1\times10^{-4}\,M$ and $1\times10^{-3}\,M$, respectively. Enzyme 18.2 µg.

6. As can be seen from Fig. 7, the enzyme showed an optimal pH of approximately 8.6–8.8, and 6.6 respectively, with Mn^{++} and Co^{++}. When Mg^{++} was used as metal activator the optimal pH of the reaction was rather broad, ranging from 6 to 8.

Specificity of the Enzyme—The puried enzyme was specific for ATP as phosphate donor and for thiamine as phosphate acceptor, and showed no activity with ADP or TMP (Table IV). This would be against a two step phosphorylation of thiamine. As regards to other nucleoside triphosphates, the enzyme has rather a broad specificity, the details of which will be treated in the succeeding paper (*6a*). Various nucleoside diphosphates tested were all inactive as phosphate donor.

The specificity of enzyme for pyrophosphate acceptor was not studied, as the present assay method was quite specific for TDP. However, the fact that NPT was a strong competitive inhibitor, while OT had only a slight inhibitory effect (Table VII), suggested that the amino group in 6-position of the pyrimidine ring would be implicated as one of the binding sites with enzyme. TDP was not formed from OMP plus Th in the presence of ATP (Table VI).

Stoichiometry of Reaction—It was rather difficult to obtain a complete balance sheet between ATP and thiamine consumed, and AMP and TDP produced, because of the extremely small amount of TDP produced during the reaction. The stoichiometric formation of AMP and TDP was shown by large-scale experiments which produced 0.31 μ mole of TDP. AMP was isolated by column chromatography on Dowex 1 and determined spectrophotometrically and calculated as 0.33 μ mole using the molecular extinction coefficient of 15.1×10^3 at 257 mμ (pH 3.0). This AMP was identified by paper chromatography and located on the paper as a single spot by ultraviolet absorption.

Inhibition of Thiaminokinase Activity—The effect of several different enzyme

TABLE V

Inhibition of Thiaminokinase Reaction

	Final concentration	Inhibition
	(*M*)	(*per cent*)
EDTA	0.1	97.4
NaCl	1.0	70.2
Phosphate	0.1	65.8
Phosphate	0.02	14.0
Arsenate	0.1	54.6
$CuSO_4$	5×10^{-4}	22.8
$CuSO_4$	5×10^{-5}	6.2
KCN	0.01	8.3
Arsenite	0.01	5.0
$(NH_4)_2SO_4$	1.0	0
NaF	0.01	36.2
AMP	1×10^{-3}	59.2
IMP	1×10^{-3}	28.2
ADP	1×10^{-3}	35.0
Adenosine	1×10^{-3}	0

Incubation was carried out under standard conditions except that 1×10^{-2} *M* $MgSO_4$ was used as activator. In these experiments the inhibitor was added to the reaction mixture before the addition of the substrates to initiate the reaction. After terminating the reaction, 0.3 ml.-aliquots were taken and assayed for TDP. Under these conditions, no inhibition was observed of the apocarboxylase system.

inhibitors is summarized in Table V. In these experiments, Mg^{++} was used as activator cation, as Mn^{++} was found to form an insoluble precipitate with some agents such as phosphate or arsenate. Besides these inhibitors, 0.01 *M* monoiodoacetic acid inhibited the reaction by 22.3 per cent, and $CuSO_4$ caused a complete inhibition at the concentration of 6.7×10^{-3} *M*. In this case, however, these two substances were also inhibitory to the apocarboxylase system (their final concentrations in the TDP assay system were one-tenth of those in thiaminokinase reaction). Their effect might not be attributed to the fact that thiaminokinase is dependent upon SH-groups for its activity, because either apocarboxylase (*23*) or thiamine (*24*) was known to bind with some of the SH-blocking agents.

AMP, a product of the reaction, inhibited the enzyme by 60 per cent at

TABLE VI

Effect of Thiamine Analogs on Reaction

Additions	Alone	With thiamine	Inhibition
	($\mu l.$ CO_2)	($\mu l.$ CO_2)	(*per cent*)
Thiamine	169.5	—	—
ET	29.3	105.4	37.8
BT	3.4	129.3	23.7
HET*	5.6	160.9	5.7
OMP	—	172.0	0
Th	—	162.0	1.8
OMP+Th	0.6	—	—

* *S*-Hydroxyethylthiamine.

The concentration of thiamine analogs was 1×10^{-3} *M*. Other conditions were those of standard assay system as described in the text.

TABLE VII

Inhibition of Thiaminokinase by NPT and OT

Additions	TDP formed	Inhibition
	($m\mu g.$)	(*per cent*)
Thiamine (5×10^{-5} *M*)	164	—
,, +OT (2.5×10^{-4} *M*)	164	0
,, +OT (1×10^{-3} *M*)	157	4.2
,, +OT (1×10^{-2} *M*)	128	21.8
Thiamine (1×10^{-4} *M*)	300	—
,, +NPT (1×10^{-4} *M*)	202	32.6
,, +NPT (5×10^{-3} *M*)	19.5	93.5

Standard conditions were used except for the concentration of thiamine and the addition of OT or NPT as indicated. Incubation at 30° for 20 minutes.

a concentration of $1\times10^{-3}\,M$. ADP was also inhibitory but to a smaller extent, while adenosine exhibited no effect on the reaction rate. On the other hand, TDP, another reaction product, was quite indifferent. Phosphate ions were inhibitory at $0.1\,M$, and the stimulating effect of low concentrations of phosphate as described by Steyn-Parvé (*3*) was not observed.

Among thiamine analogs tested, NPT and OT were competitive inhibitors of the reaction, the former being much more effective (Table VII). K_i values of 0.85×10^{-4} and $1\times10^{-2}\,M$ were obtained from Lineweaver Burk plots for NPT (Fig. 5) and OT, respectively. ET also exhibited an appreciable inhibition (Table VI), the nature of which may be competitive as was observed in the case of animal thiaminokinase (*5*). BT was moderately inhibitory. Neither OMP nor Th had any inhibitory effect on the reaction.

DISCUSSION

The partial purification of thiminokinase was previously reported by Steyn-Parvé (*3*), who stated that the enzyme was extracted only by freezing and thawing of the fresh baker's yeast followed by maceration at 37°, as drying and subsequent maceration of the yeast entailed considerable loss of the enzyme activity. In the present study, the enzyme was successfully extracted from air-dried baker's yeast, the autolyzate of which was as active as Steyn-Parvé's extracts. Plasmolysis of fresh baker's yeast with ether-dry ice (*25*) or by freezing and thawing (*3*) followed by maceration usually gave preparations with a specific activity of less than 1 unit per mg. protein. Therefore, it was decided to use dried baker's yeast as the starting material. The purified enzyme showed a specific activity 8–12 times greater than that of Steyn-Parvé's.

Alkaline washed yeast (etiozymase) has so far been used by many investigators to determine TDP. This was not a reliable source of apocarboxylase as was already discussed in the previous paper (*13*). Although the apocarboxylase preparation used in this experiment contained a residual thiaminokinase activity, it did not interfere with the assay system, because, under the conditions for TDP determination, ATP was completely inactive as phosphate donor. When GTP or ITP was used as phosphate donor, it was possible to eliminate the thiaminokinase activity completely by the addition of NPT to the TDP assay system.

It may be pertinent to discuss here about the inhibition of the enzyme activity by NPT. According to Eusbi and Cerecedo (*26*), yeast thiaminokinase was inhibited by OT but not by NPT. They stated that the results were diametrically opposed to those obtained with the rat liver enzyme, which was inhibited by NPT but not by OT (*26*, *27*). The present results indicated, however, that NPT was also an inhibitor with yeast enzyme as potent as in the case of animal enzyme, whereas OT showed only a slight inhibitory effect. It is not known whether these thiamine analogs produce their inhibition simply by competing for enzyme surface or whether they can actually be phosphorylated to produce the diphosphate forms. In the case of ET, it has not only an inhibitory effect when added with thiamine, but it seems to be converted to a substance which shows a little TDP activity (Table VI). ET may be phosphorylated to its diphosphate form, which has a similar activity to TDP, although to a very small extent.

Since the first study of Weil-Malherbe *(1)*, thiaminokinase reaction has been recognized to produce very little amount of TDP, the reason for which remained to be explained. In fact, the amount of TDP produced does not exceed a few per cent of that of substrate added under the standard conditions for assay. It might not be due to the decrease in ATP or thiamine, but the limiting factor is probably the amount of thiaminokinase protein itself. An experiment with a larger amount of enzyme and smaller amount of substrate seems to support this view. In this experiment, 15 mμ moles of thiamine was incubated with 1.5 μ moles of ATP in the presence of about 4 mg. of partially purified thiaminokinase at 30° for 60 minutes. During this incubation period, 9.2 mμ moles of TDP was formed, which corresponded to about 60 per cent of the thiamine added. A better yield is expected when incubation is carried out for a longer time.

It has been known that many of the enzyme systems which utilize ATP have an absolute requirement for divalent cations as cofactor *(28)*. Thus, it appears that the activating ion may exert its effect by forming a complex with nucleotide and the actual substrate for the reaction is considered to be this complex and not free ATP and metal ions *(29–33)*. The dissociation constant of this specific complex would differ with the species of cations and be affected by external conditions such as the concentrations of activator ions or species and pH of the buffer system used. The difference in these factors would be reflected on the rate of the reaction. Among a number of cations tested, Mg^{++}, Mn^{++} and Co^{++} were most effective (Table III). These three activating ions are all divalent cations with a coordination number of 6, and have a strong tendency to form chelates. As shown in Fig. 6, an excess of Mn^{++} and Co^{++} was strongly inhibitory at pH 8.6, while Mg^{++} showed no inhibitions up to the concentration of 5×10^{-2} *M*. A possible explanation for these results is that the real substrate for the reaction is metal-ATP complex and the addition of excess metal might results in a decrease of the concentration of active substrate by forming a different chelate form containing a greater number of metal, such as $(metal)_2$-ATP in stead of metal-ATP. Mg^{++}, on the other hand, owing to its relatively weak tendency to form chelates, does not combine further with metal-ATP and is not inhibitory even when it is present in greater excess. At pH 7.0, none of these three cations inhibit the reaction, because ATP is not completely in dissociated form at this pH, and it could not bind with an additional metal ion to form $(metal)_2$-ATP which would be inactive or inhibitory substance in this reaction. The inhibition caused by a high concentration of Na (Table V) could also be explained as a result of the formation of a complex such as Na-ATP which might compete with the specific complex form. On the other hand, EDTA, phosphate, and arsenate have a strong tendency to combine with activator cations, thus competing with ATP.

The dissociation constant of the metal-ATP complex would be influenced by pH of the reaction medium. Fig. 7 shows that the optimal pH differs with different cations used. The optimal pH seems to depend upon the relative concentrations of ATP and activator cations. When metal ions are present in excess of the amount required for the maximum rate of the reaction, the pH optimum seems to shift towards acidic side.

It has been noted by several investigators (*29–33*) that an optimum ratio exists between the concentrations of activating ion and nucleotide as substrate. Hers (*29*) repoted that with the liver fructokinase the optimum concentration of Mg^{++} was equivalent to ATP, and this value was also reaffirmed by other workers (*30–33*). However, this value is by no means correct in all the cases because no linear proportionality exists between the amount of metal and ATP. In the present study, as shown in Fig. 8, the optimal concentrations of Mn^{++} were 3×10^{-3}, 1×10^{-3}, and 5×10^{-4} *M*, for 1×10^{-3}, 1×10^{-4}, and 1×10^{-5} *M* of ATP, respectively.

The transfer of an intact pyrophosphate group from ATP to thiamine, was suggested (*1*, *4*) as the reaction mechanism of this enzyme. In the present study, as represented in Table IV, the inability of ADP to substitute for ATP and the inactivity of TMP to replace thiamine also suggested that a direct pyrophosphrylation of thiamine by ATP is highly probable. The author succeeded in demonstrating this mechanism with the use of ATP-γ-P^{32} and the details of the experiments were given elsewhere (*7*).

It has recently been postulated by Nose *et al.* (*34*) that TDP is synthesized from OMP-diphosphate and Th-phosphate without ATP in baker's yeast. This might exclude free thiamine as intermediate for TDP synthesis. However, the purified thiaminokinase had no ability to produce TDP from OMP and Th in the presence of ATP, and both fragments of thiamine molecule seem to have no affinity to the enzyme, as they did not affect the reaction rate. Furthermore, with the cell-free extract obtained by plasmolysis of fresh yeast, thiamine was shown to be a much better substrate than OMP plus Th. From these findings, it might be assumed that the main route for TDP synthesis in living yeast involved the intermediate formation of thiamine.

SUMMARY

1. An enzyme which catalyzes the synthesis of TDP from thiamine was purified about 100-fold from the extract of dried baker's yeast. The purified enzyme did not contain either adenylate kinase or NDPkinase activity.

2. The main product of the reaction was identified as TDP. TMP was not formed during the reaction.

3. The Michaelis constant, K_m, of the reaction was determined respectively as 1.0×10^{-4} *M* and 0.75×10^{-4} *M* for ATP and thiamine.

4. The reaction has an absolute requirement for the presence of divalent cations as activator. Among the number of cations tested, Mn^{++}, Mg^{++}, and Co^{++} were the most active. The effect of these three metal ions on this reaction was compared under various conditions and a possible role of the metal-ATP complex in this reaction was discussed.

5. The enzyme showed the maximal activity at pH 8.6 in the presence of $2\times10^{-3}\,M$ manganese ion.

6. The purified enzyme was specific for ATP and other nucleoside triphosphates as phosphate donor and active with thiamine as phosphate acceptor, whereas ADP or TMP was inactive as substrate. The transfer of an intact pyrophosphoryl group was postulated as the reaction mechanism.

7. Among thiamine analogs tested, NPT and ET strongly inhibited the reaction, while OT showed only a slight inhibition.

The author wishes to express the heartful gratitude to Prof. N. Shimazono for his helpful advices and continuous encouragement.

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The Journal of Biochemistry, Vol. 45, No. 11, 1959

STUDIES ON α- AND ε-LYSINE ACYLASE PRODUCED BY PSEUDOMONAS SP.

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(Received for publication, May 11, 1959)

In the previous paper (*1*) the author reported that KT-83, a strain belonging to *Pseudomonas* sp. isolated from soil by Kameda *et al.* (*2*), contained two kinds of enzymes, α-acylase and ε-acylase, and that the former hydrolyzed the α-acylamino group of L-lysine and the latter the ε-acylamino group thereof. In that paper the author also described the isolation of ε-acylase from the cell of KT-83 and the mechanism of asymmetrical hydrolysis of N^{α},N^{ε}-diacyl-DL-lysine by the two enzymes.

In the present work, α-acylase was isolated from the same organism and its properties were compared with those of ε-acylase. Further, for the study of the specificity of these enzymes, a variety of acylamino acids were synthesized and their hydrolizabilities by both enzymes were compared. As a result, the author proposes a hypothesis that α-acylase combines with substrates at two points and ε-acylase at three points.

EXPERIMENTAL AND RESULTS

Preparation of Substrates

Synthesis of DL-*Ornithine Derivatives*—N^{δ}-Benzoyl-DL-ornithine: 2.0 g. of DL-ornithine hydrobromide was first converted to its copper salt with copper carbonate, then treated with benzoylchloride, and finally the copper was removed by hydrogen sulfide (*3*). The product, after recrystallization from water, showed a melting point of 254°. The yield was 1.8 g.

Analysis: Calcd. for $C_{12}H_{16}O_3N_2$: C 61.00; H 6.83; N 11.86
Found : C 60.89; H 7.01; N 11.89.

N^{α},N^{δ}-Dibenzoyl-DL-ornithine: 1.5 g. of DL-ornithine hydrobromide was benzoylated with sodium hydroxide and benzoylchloride (*4*). The product, after recrystallization from a mixture of alcohol and water, showed a melting point of 185°. The yield was 1.5 g.

Analysis: Calcd. for $C_{19}H_{20}O_4N_2$: C 67.04; H 5.92; N 8.23
Found : C 66.95; H 5.88; N 8.23.

Synthesis of DL-*Lysine Derivatives*—N^{ε}-Acetyl-DL-lysine (I): 2.0 g. of DL-lysine sulfate was converted to its copper salt with copper carbonate, then the salt was acetylated with barium hydroxide and acetic anhydride, and finally the copper was removed with hydrogen sulfide (*5*). The product melted at 256–7° after recrystallization from a mixture of water and alcohol. The yield was 0.7 g.

Analysis: Calcd. for $C_8H_{16}O_3N_2$: C 51.05; H 8.57; N 14.88
Found : C 51.13; H 8.73; N 14.88.

N^{α},N^{ε}-Diacetyl-DL-lysine (II): an aqueous solution of 3.0 g. of DL-lysine hydrochloride was passed through a column of ion exchange resin, IR-120, to remove the hydrogen chloride and the free DL-lysine thus produced was acetylated at 50° with glacial acetic acid and acetic anhydride. The product melted at 140° after recrystallization from a mixture of methanol and ethyl acetate. The yield was 2.8 g.

Analysis: Calcd. for $C_{10}H_{18}O_4N_2$: C 52.16; H 7.88; N 12.17
Found : C 52.15; H 7.59; N 12.38.

N^{ε}-Benzoyl-DL-lysine (III): this compound was prepared from 2.0 g. of DL-lysine hydrochloride by the same method as for the synthesis of N^{δ}-benzoyl-DL-ornithine. When recrystallized from water, the product melted at 267°. The yield was 1.9 g.

Analysis: Calcd. for $C_{13}H_{18}O_3N_2$: C 62.38; H 7.25; N 11.19
Found : C 62.60; H 7.28; N 11.24.

N^{α},N^{ε}-Dibenzoyl-DL-lysine (IV): this compound was produced from 1.5 g. of DL-lysine hydrochloride by the same method as that employed for the preparation of N^{α},N^{δ}-dibenzoyl-DL-ornithine. The product was purified by recrystallization from a mixture of alcohol and water, m.p. 145°. The yield was 1.6 g.

Analysis: Calcd. for $C_{20}H_{22}O_4N_2$: C 67.78; H 6.26; N 7.91
Found : C 67.65; H 6.31; N 7.95.

N^{α}-Acetyl-N^{ε}-benzoyl-DL-lysine (V): 2.0 g. of N^{ε}-benzoyl-DL-lysine (III) was heated with glacial acetic acid and acetic anhydride (*6*) and the product was recrystallized from water, m.p. 143°. The yield was 1.3 g.

Analysis: Calcd. for $C_{15}H_{20}O_4N_2$: C 61.63; H 6.90; N 9.58
Found : C 61.53; H 6.83: N 9.30.

N^{α}-Formyl-N^{ε}-benzoyl-DL-lysine (VI): 5.0 g. of N^{ε}-benzoyl-DL-lysine (III) was heated with formic acid and acetic anhydride at 30° (*6*) and the product was recrystallized from water, m.p. 167°. The yield was 0.7 g.

Analysis: Calcd. for $C_{14}H_{18}O_4N_2$: C 60.42; H 6.52; N 10.07
Found : C 60.78; H 6.60; N 9.98.

N^{ε}-Carbobenzoxy-DL-lysine (VII): 4.5 g. of DL-lysine hydrochloride was first converted to its copper salt with copper carbonate, then the salt was treated with sodium hydroxide and carbobenzoxychloride, and finally the copper was removed with hydrogen sulfide (*5*). The product was recrystallized from water, m.p. 254–5°. The yield was 3.2 g.

Analysis: Calcd. for $C_{14}H_{20}O_4N_2$: C 59.98; H 7.19; N 9.99
Found : C 60.32; H 7.16; N 10.20.

N^{α}.Benzoyl-N^{ε}-carbobenzoxy-DL-lysine (VIII): 3.0 g. of N^{ε}-carbobenzoxy-DL-lysine was benzoylated with sodium hydroxide and benzoylchloride (*7*) and the product was recrystallized from a mixture of alcohol and water, m.p. 133°. The yield was 2.8 g.

Analysis: Calcd. for $C_{21}H_{24}O_5N_2$: C 65.61; H 6.29; N 7.29
Found : C 65.61; H 6.16; N 7.31.

N^{α}-Benzoyl-DL-lysine (IX): 1.9 g. of N^{α}-benzoyl-N^{ε}-carbobenzoxy-DL-lysine (VIII) was dissolved in methanol containing hydrochloric acid and the solution was subjected to catalytic reduction in the presence of palladium-carbon (*8*). The crude product was purified by recrystallization from water, m.p. 213°. The yield was 0.9 g.

Analysis: Calcd. for $C_{13}H_{18}O_3N_2 \cdot H_2O$: C 58.21; H 7.46; N 10.44
Found : C 58.48; H 7.49; N 10.55.

N^{ε}-Benzoyl-DL-lysine amide hydrochloride (X): 5.0 g. of N^{ε}-benzoyl-DL-lysine was esterified with anhydrous methanol and dry hydrogen chloride. The product, being hardly crystallizable, was immediately dissolved in methanol and the solution, after being saturated with ammonia, was heated in an autoclave at 60° for 10 hours. The product was recrystallized from methanol, m.p. 241°. The yield was 3.6 g.

Analysis: Calcd. for $C_{13}H_{19}O_2N_3 \cdot HCl$: C 54.64; H 7.00; N 14.71; Cl 12.43
Found : C 54.90; H 7.05; N 14.25; Cl 11.74.

N^{α}-Benzoyl-DL-lysine amide hydrochloride (XI): 2.0 g. of N^{α}-benzoyl-N^{ε}-carbobenzoxy-DL-lysine (VIII) was esterified with diazomethane to give the corresponding ester in the form of a white resin. The ester was treated as in the preparation of N^{ε}-benzoyl-DL-lysine amide hydrochloride (X) and the product was recrystallized from a mixture of methanol and ether, m.p. 141°. The yield was 1.5 g.

The amide thus produced was treated in the same manner as in the synthesis of N^{α}-benzoyl-DL-lysine (IX) (*9*) and the product was recrystallized from a mixture of methanol and ethyl acetate, m.p. 239°. The yield was 1.2 g.

Analysis: Calcd. for $C_{13}H_{19}O_2N_3 \cdot HCl \cdot \frac{1}{2}H_2O$: C 52.97; H 7.13; N 14.26; Cl 12.05
Found : C 52.83; H 6.92; N 14.18; Cl 12.18.

N^{ε}-Acetyl-DL-lysine amide hydrochloride (XII): 5.0 g. of N^{ε}-acetyl-DL-lysine (I) was amidated by the same method as that used for the preparation of N^{ε}-benzoyl-DL-lysine amide hydrochloride (X) and the product was recrystallized from methanol, m.p. 242°. The yield was 3.2 g.

Analysis: Calcd. for $C_8H_{17}O_2N_3 \cdot HCl$: C 42.95; H 8.11; N 18.79; Cl 15.85
Found : C 42.88; H 8.23; N 18.69; Cl 15.77.

N^{α},N^{ε}-Dibenzoyl-DL-lysine amide (XIII): 2.0 g. of N^{α},N^{ε}-dibenzoyl-DL-lysine (IV) was esterified with diazomethane and the product was recrystallized from a mixture of methanol and ether, m.p. 113°. The yield was 1.7 g. The ester was then amidated by the conventional method and recrystallized from methanol, m.p. 206°. The yield was 1.3 g.

Analysis: Calcd. for $C_{20}H_{23}O_3N_3$: C 67.97; H 6.56; N 11.89
Found : C 67.89; H 6.85; N 11.62.

Synthesis of DL-*Homolysine and Its Derivatives*—DL-Homolysine hydrochloride: 17.0 g. of benzoyl-ε-chloroamylamine (*10*) prepared from dihydropyrane was dissolved in a sodium ethoxide solution produced from 2.5 g. of metallic sodium and 75 ml. of absolute ethanol and the solution was heated with 31.0 g. of ethyl acetoaminomalonate for 20 hours. The solvent was distilled off from the reaction mixture and the residue, after addition of 50 ml. of water, was extracted with ether. The extract was dried, the ether was distilled off, and the residue was boiled gently for 2 hours with 50 ml. of 20 per cent potassium hydroxide solution. The reaction mixture was shaken with ether to remove impurities and the aqueous solution, after being acidified with hydrochloric acid, was extracted with ethyl acetate. The solvent was distilled off from the extract, the residue was boiled with 50 ml. of 6 *N* HCl, and pyridine was added to the concentrated reaction mixture, whereupon crystals separated out (*11*). The product melted at 271°* after recrystallization from aqueous alcohol. The yield was 6.7 g.

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* The melting point of this compound given in the literature is 176°.

Analysis: Calcd. for $C_7H_{16}O_2N_2 \cdot HCl$: C 42.79; H 8.72; N 14.26; Cl 18.07
Found : C 42.57; H 8.74; N 14.51; Cl 18.16.

N^{ζ}-Benzoyl-DL-homolysine: as in the case of N^{δ}-benzoyl-DL-ornithine, 2.0 g. of DL-homolysine hydrochloride was first converted to its copper salt with copper carbonate, then the salt was benzoylated with benzoylchloride, and finally the copper was removed with hydrogen sulfide. The product melted at 253° after recrystallization from water. The yield was 1.8 g.

Analysis: Calcd. for $C_{14}H_{20}O_3N_2$: C 63.61; H 7.63; N 10.60
Found : C 63.00; H 7.66; N 10.69.

N^{α},N^{ζ}-Dibenzoyl-DL-homolysine: 1.5 g. of DL-homolysine hydrochloride was benzoylated by the usual method with sodium hydroxide and benzoylchloride. The product was recrystallized from aqueous alcohol, m.p. 158°. The yield was 1.5 g.

Analysis: Calcd. for $C_{21}H_{24}O_4N_2$: C 68.46; H 6.57; N 7.60
Found : C 68.42; H 6.55; N 7.54

Assay of the Enzyme

For the enzyme assay incubation was carried out under conditions described in the legends of the tables and figures and the hydrolysis products were determined by the method reported previously (*1*) and they were also identified by electrophoresis. For the substrates which were hardly hydrolyzed under the above conditions, the incubation time was prolonged up to 24 hours.

Preparation of α- and ε-Acylase from KT-83

A bouillon culture medium was inoculated with KT-83 at pH 7.2, and incubated at 25° for 24 hours aerobically. About 150–200 g. of the wet cell was obtained from 10 liters of the culture. The cell separated by centrifugation was ground with alumina and extracted with 400 ml. of 0.02 *M* phosphate buffer at pH 7.8. Preparation of ε-acylase from the extract which contained α- as well as ε-acylase was conducted by the method reported in the previous paper (*1*).

Preparation of α-acylase from the extract was carried out as follows.

The extract was adjusted to pH 4.5 and the resulting copious precipitate, after being separated by centrifugation, was dissolved in 100 ml. of 0.02 *M* phosphate buffer at pH 7.8, and 4 g. of streptomycin sulfate was added gradually in the solution with stirring. The resulting precipitate was centrifuged, the clear supernatant was mixed with the same volume of a saturated aqueous solution of ammonium sulfate with stirring, and the separated precipitate was collected. The precipitate was dissolved in a little water and the solution was dialyzed against cold water for 24 hours. The solution was then passed through a column packed with a mixture of 15 g. of Celite 535 and 5 g. of calcium phosphate and the column, after being washed well with water, was eluted with about 120 ml. of a 0.5 *M* aqueous solution of ammonium sulfate. The eluate was again dialyzed against cold water and then lyophilized to give about 750 mg. of a white powdery product. The product contained no ε-acylase and showed a specific activity against N^{α}, N^{ε}-dibenzoyl-L-lysine of 24.5 μ moles/hour/mg. of protein (Table I).

Properties of α- and ε-Acylase of KT-83

Optimum pH of α-acylase was 7.8 and that of ε-acylase was 6.0, and their optimum temperature was 37–38°. ε-Acylase was markedly activated

TABLE I

Purification of the α-Acylase

Stage of purification	Total protein (*mg.*)	α-Acylase activities1) (*μM/hr.*)	Yield (%)	Specific activity (*μM/hr./mg. protein*)	Total ε-acylase activities1) (*μM/hr.*)
Cells, wet weight 200 g.		48,000	100		157,000
Ground with alumina and extracted with buffer at pH 7.8 400 ml.	107,200	33,200	69.5	1.27	115,600
Precipitate formed at pH 4.5 was dissolved in 100 ml. phosphate buffer at pH 7.8	5,400	22,090	45.9	4.09	3,240
Streptomycin sulfate was added, and its supernatant was fractionated with ammonium sulfate (0–50%sat.)	1,060	17,300	35.8	16.3	—
Fraction purified by treating with Ca-phosphate	620	15,200	31.3	24.5	—

1) Digests for the α-acylase contained 2.5 ml. of 0.02 *M* N^{α},N^{ε}-dibenzoyl-*DL*-lysine plus 2.5 ml. of 0.02 *M* phosphate buffer at pH 7.8 containing the enzyme and the digests for the ε-acylase contained 2.5 ml. of 0.02 *M* N^{ε}-benzoyl-*DL*-lysine plus 2.5 ml. of 0.02 *M* phosphate buffer at pH 6.0 containing the enzyme.

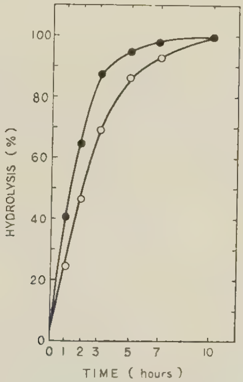

FIG. 1. Hydrolysis of benzoyl-L-lysines by the α-acylase (—○—) and the ε-acylase (—●—). Digest consisted of 2.5 ml. of 0.02 *M* N^{α},N^{ε}-dibenzoyl-DL-lysine plus 2.5 ml. of 0.02 *M* phosphate buffer at pH 7.8 containing 0.002 *M* Co^{++} and 0.5 mg. of the α-acylase. Digests contained 2.5 ml. of 0.02 *M* N^{ε}-benzoyl-DL-lysine plus 2.5 ml. of 0.1 *M* citrate buffer at pH 6.0 containing 0.002 *M* Zn^{++} and 0.05 mg. of the ε-acylase.

by Zn^{++} as described in the previous report (*1*).

Study was made on the relation of the incubation time and concentration of substrates to the activity of the enzymes. Up to the first 3 hours the amount of every substrate hydrolyzed was proportional to the length of time (Fig. 1), and the hydrolysis was almost quantitative when the substrate concentration was lower than 0.02 *M*. K_m value of α-acylase for N^α,N^ϵ-dibenzoyl-DL-lysine was found to be about 6.2×10^{-2} *M* and that of ε-acylase for N^ϵ-benzoyl-DL-lysine about 7.1×10^{-2} *M*. However, as N^ϵ-benzoyl-DL-lysine was insoluble at concentrations higher than 0.15 *M*, the K_m value should be regarded as approximative.

TABLE II

Effect of Various Ions on α-Acylase

Ion (10^{-3} *M*)	Hydrolysis of N^α,N^ϵ-dibenzoyl-L-lysine[1] (%)
Fe^{+++}	18.0
Ni^{++}	23.0
Ca^{++}	18.0
Fe^{++}	19.0
Mn^{++}	20.0
Mg^{++}	30.0
Co^{++}	92.0
Zn^{++}	17.0
EDTA	18.0
Ascorbic acid	37.0
KCN	40.0
None	18.0

1) Digests contained 2.5 ml. of 0.02 *M* N^α,N^ϵ-dibenzoyl-DL-lysine plus 2.5 ml. of 0.02 *M* veronal buffer at pH 7.8 containing various ions and 0.5 mg. of the purified enzyme; incubation time, 1 hour.

TABLE III

Activation of α-Acylase by Co^{++} of Varying Concentration

	Co^{++} at concentration of					
	0	1×10^{-4} *M*	2×10^{-4} *M*	1×10^{-3} *M*	2×10^{-3} *M*	1×10^{-2} *M*
N^α,N^ϵ-dibenzoyl-L-lysine hydrolyzed[1] (%)	15.0	35.0	50.0	75.0	60.0	40.0

1) Digests contained 2.5 ml. of 0.02 *M* N^α,N^ϵ-dibenzoyl-DL-lysine plus 2.5 ml. of 0.02 *M* phosphate buffer at pH 7.8 containing various amounts of the Co^{++} and 0.5 mg. of the purified enzyme; incubation time, 1 hour.

Influence of various ions on the activity of the purified α-acylase was investigated. Fe^{++}, Ni^{++}, Ca^{++}, Mn^{++}, Mg^{++}, Co^{++}, Zn^{++} and Fe^{+++} in the form of sulfate or chloride, and EDTA, ascorbic acid and potassium cyanide were investigated at 10^{-3} *M* final concentration in veronal buffer at pH 7.8, respectively. It was found that like α-acylase from hog kidney (*12*) and *Aspergillus* (*13*) the enzyme was strongly activated by Co^{++} (Table II), with the highest activation at 10^{-3} *M*, *e.g.* the hydrolysis of N^{α},N^{ε}-dibenzoyl-L-lysine being increased by 420 per cent (Table III).

TABLE IV

Substrate Specificity of α-, and ε-Acylase

Substrate	α-Acylase	ε-Acylase
	(*μM/hr./mg. protein*)	(*μM/hr./mg. protein*)
Ornithine derivatives		
N^{δ}-benzoyl-DL-ornithine	0	0
N^{α},N^{δ}-dibenzoyl-DL-ornithine	23.2	0
Lysine derivatives		
N^{ε}-acetyl-DL-lysine	0	157
N^{ε}-acetyl-DL-lysine amide HCl	0	0
N^{ε}-benzoyl-DL-lysine	0	892.0
N^{ε}-benzoyl-DL-lysine amide HCl	0	0
N^{ε}-carbobenzoxy-DL-lysine	0	785.0
N^{α}-benzoyl-DL-lysine	20.5	0
N^{α}-benzoyl-DL-lysine amide HCl	0	0
N^{α},N^{ε}-dibenzoyl-DL-lysine	24.0	0
N^{α},N^{ε}-dibenzoyl-DL-lysine amide[1]	0	0
N^{α},N^{ε}-diacetyl-DL-lysine	2.3	0
N^{α}-formyl-N^{ε}-benzoyl-DL-lysine	1.5	0
N^{α}-acetyl-N^{ε}-benzoyl-DL-lysine	1.5	0
N^{α}-benzoyl-N^{ε}-carbobenzoxy-DL-lysine	13.5	0
Homolysine derivatives		
N^{ζ}-benzoyl-DL-homolysine	0	0
N^{ζ},N^{δ}-dibenzoyl-DL-homolysine	32.5	0

Digest consisted of 2.5 ml. of 0.02 *M* substrate plus 2.5 ml. of 0.02 *M* phosphate buffer at pH 7.8 containing 0.002 *M* Co^{++} and 0.5 mg. of the α-acylase; Digests contained 2.5 ml. of 0.02 *M* substrate plus 2.5 ml. of 0.1 *M* citrate buffer at pH 6.0 containing 0.002 *M* Zn^{++} and 0.05 mg. of the ε-acylase.

1) Incubated as suspension.

α-Acylase migrated toward the anode when subjected to paper electrophoresis (pH 7.5; μ 0.05) for two hours.

Specificity—For a variety of the aminoacyl derivatives of ornithine, lysine and homolysine the action of the purified α- and ε-acylase was tested respectively. The results are presented in Table IV.

DISCUSSION

In the previous paper (*1*) the author reported that α- and ε-acylases produced by a strain, KT-83, of *Pseudomonas* sp., hydrolyzed L-forms of acylamino acids but not D-forms.

The following conclusion was drawn from the result of the study on the properties of these enzymes, which was conducted referring to the study of the specificity of exopeptidase (*14–16*), an enzyme closely resembling the above ones.

α-Acylase hydrolyzes acylamino groups only at the α-position of amino acids, and in this case it hydrolyzes aromatic acyl groups such as carbobenzoxy and benzoyl groups more readily than aliphatic groups such as formyl and acetyl groups. This may be due to stronger affinity of the enzyme to aromatic groups than to aliphatic groups. However, the structure of the ε-amino group, whether free or combined with an aromatic or an aliphatic group, has no influence on the hydrolysis. From this fact it seems likely that the substituents beyond the β-carbon atom do not exert direct influence on the hydrolysis. Hence, α-acylase hydrolyzes acylamino groups at the α-position of such amino acids as ornithine, lysine, homolysine, phenylalanine, phenylglycine (*17*), leucine (*18*), glutamic acid (*19*), methionine (*20*), *etc.* This is supported by the fact that the α-acylase of hog kidney (*21*) and *Aspergillus* (*22*), hydrolyzes α-acylamino groups of various amino acids. The ease of hydrolysis, however, differs depending on the nature of the substrates. This fact seems to suggest that the structure beyond the β-carbon atom also takes part, though slightly and indirectly, in the hydrolysis. Amides of various amino acids are never hydrolyzed by α-acylase. This indicates that this enzyme requires a free carboxyl group for displaying its activity.

Smith *et al.* (*23*) formerly proposed a hypothesis on the mechanism of the action of exopeptidase. Following this hypothesis the present author assumed the activation of α-acylase by Co^{++} and the combination of the activated enzyme with substrates at their carboxyl and α-acylamino groups (Fig. 2).

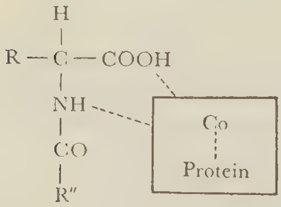

FIG. 2. Hypothetical combination of substrate with α-acylase.

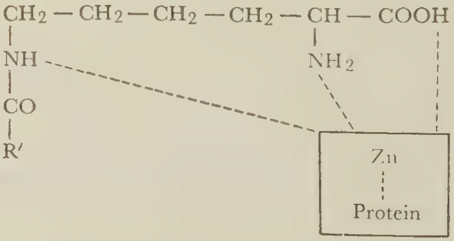

FIG. 3. Hypothetical combination of substrate with ε-acylase.

This supposition appears to explain why the structure beyond the β-carbon atom has but little influence on the hydrolysis.

On the other hand, ε-acylase hydrolyzes only acylamino groups at the ε-position of amino acids, and like α-acylase its hydrolyzing ability is stronger to aromatic acyl groups than to aliphatic ones. In this case, if the α-amino group is acylated, ε-acylamino groups are no longer hydrolyzed. This indicates that ε-acylase requires a free amino group at the α-position for its activity. Further, when the carboxyl group is amidated, ε-acylamino groups are also no longer hydrolyzed. This suggests that ε-acylase requires both free amino group and free carboxyl group to exhibit its activity. Therefore, the combination of this enzyme with substrates seems to occur at three points (Fig. 3).

These considerations would explain why ε-acylase does not hydrolyze the acylamino groups of N^δ-acyl-L-ornithine and N^ζ-acyl-L-homolysine, its hydrolyzing ability being limited only to ε-acylamino groups of L-lysine.

SUMMARY

1. α-Acylase, hydrolyzing only acyl groups at the α-amino group of L-lysine and ε-acylase, hydrolyzing only acyl groups at the ε-amino group, were isolated from a strain (KT-83) of *Pseudomonas* sp., and their properties were examined. Optimum pH of α-acylase was 7.8 and that of ε-acylase was 6.0, and the optimum temperature was 37-38° for both enzymes. The former enzyme was activated by Co^{++} and the latter by Zn^{++}, and the optimum concentration of the ions was 1×10^{-3} *M*.

2. For the study of the specificity of these enzymes, derivatives of DL-ornithine, DL-lysine and DL-homolysine were synthesized.

3. It was found that α-acylase requires a free carboxyl group at the α-position of substrates for the activity and ε-acylase a free amino group at the α-position, in addition to a free carboxyl group. Both enzymes acted upon aromatic acyl groups more readily than upon aliphatic ones, but, while α-acylase attacked α-acylamino groups of various amino acids, the activity of ε-acylase was limited only to ε-acylamino groups of lysine. These results may be explained by assuming that α-acylase combines with substrates at two points and ε-acylase at three points.

The author wishes to express his appreciation to Dr. S. Kuwada, Dr. S. Tatsuoka and Dr. Y. Kameda for their great favour and continuous interest.

Thanks are also due to Dr. Y. Ueno, Mr. A. Miyake, Dr. E. Ohmura and Dr. K. Ogata for their support, and to Mr. I. Imada and Mr. K. Tomoda for their technical help.

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NOTES

The Journal of Biochemistry, Vol. 46, No. 11, 1959

THE SYNTHESIS OF STERO-BILE ACIDS

XXXIII. ON THE STRUCTURE OF TRIHYDROXY-ISOSTEROCHOLENIC ACID

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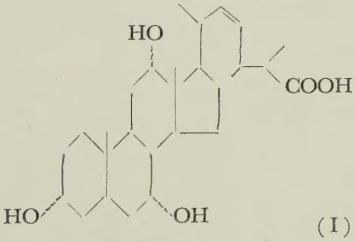
(Received for publication, April 20, 1959)

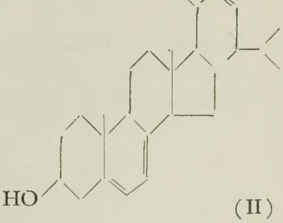
As previously reported by the author (*1*), $3\alpha,7\alpha,12\alpha$-trihydroxy-24-methylcoprostanic acid can be synthesized from norcholylmethylketone and ethyl α-bromopropionate by modified Reformatsky reaction. This acid has been assumed to be identical with trihydroxy-isosterocholanic acid derived from natural trihydroxy-isosterocholenic acid, on the basis of the determination of the mixed melting point. But a further evidence for the identity of both acids has not yet been obtained.

The present paper is concerned with the infrared absorption spectra of methyl trihydroxy-isosterocholenate, methyl trihydroxy-isosterocholanate, and methyl trihydroxy-24-methylcoprostanate. The results are shown in Fig. 1.

In the infrared spectrum of methyl trihydroxy-isosterocholenate, a strong band was found at 10.30 μ, so it is clear that this ester contains a *trans* 22–23 double bond (*2*). Comparison with infrared spectra of methyl trihydroxy-isosterocholanate and methyl trihydroxy-24-methylcoprostanate makes up clear that both esters are identical. From this result, it is assumed that trihydroxy-isosterocholenic acid and Δ^{22}-$3\alpha,7\alpha,12\alpha$-trihydroxy-24-methylcoprostenic acid (I) are the same substance.



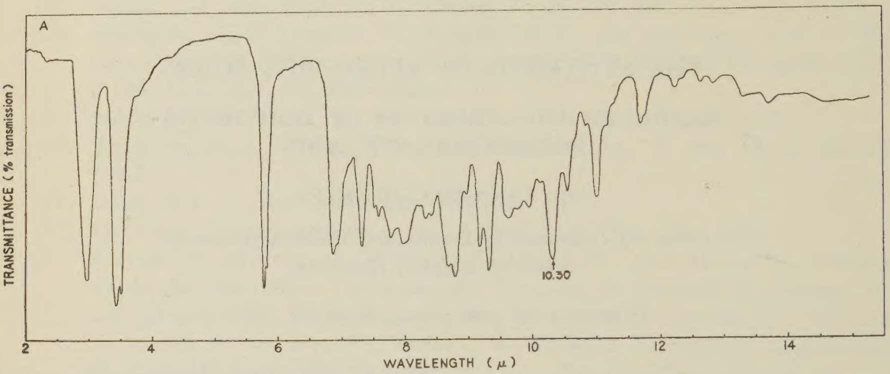
(I)



(II)

Although the biosynthetic process of bile acids has not yet been disclosed, the stero-bile acids and bile-sterols may represent the intermediates from sterols to C_{24} bile acids. If bile acid is arising from ergosterol by ω-oxidation of the side chain, by which trihydroxy-coprostanic acid may presumably be derived from cholesterol (*3*), the produced bile acid may have a side chain of a structure such as $-CH(CH_3)-CH=CH-CH(CH_3)-CH(CH_3)-COOH$.

It is reasonable to suppose that trihydroxy-24-methylcoprostenic acid (I) having such a side chain may be formed from ergosterol (II).



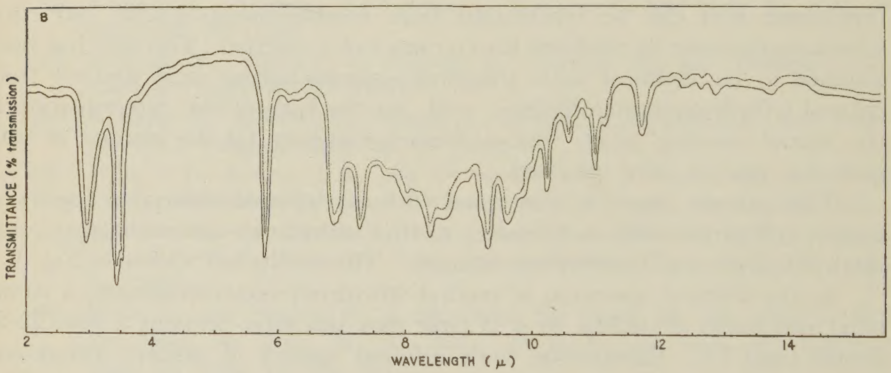


FIG. 1. Infrared spectra of methyl trihydroxy-isosterocholenate (A), methyl trihydroxy-isosterocholanate ——; and methyl trihydroxy-24-methylcoprostanate ----, (B), KBr disk.

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昭和 32 年 7 月 9 日 第三種 郵便物 認可
第46巻 第11号 昭和34年11月25日発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 46, No. 11, November 25, 1959

Vol. 46 **No. 11**

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